THE SYNTHESIS AND APPLICATION OF PHOTOLABILE POLYNUCLEOTIDES

A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy at the University of Warwick

by

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December, 1978

..... to Sue .....

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Finally, I wish to pay tribute to my wife, Sue, without whose continual encouragement, aid and support this thesis would not have been completed.

DECLARATION

The work described in this thesis is the original work of the author, except where acknowledgement is made to work and ideas previously published. It was carried out in the Department of Chemistry and Molecular Sciences and the Department of Biological Sciences, University of Warwick, between October, 1975 and September, 1978, and has not been submitted previously for a degree at any institution.

Publications

Parts of the research described in this thesis have appeared in the scientific literature as follows:-

1. The reaction between thiols and 8-azido adenosine derivatives
   Cartwright, I.L., Hutchinson, D.W. and Armstrong, V.W. (1976) Nucleic Acids Research, 3, 2331-9.

2. A simple, rapid preparation of α-[^{32}P]-labelled adenosine diphosphate
   Cartwright, I.L. and Hutchinson, D.W. (1977) Nucleic Acids Research, 4, 2507-10.

ABBREVIATIONS

Abs	Absorbance
Ado	Adenosine
br^{8}AMP	8-bromo adenosine monophosphate
Brd Urd	Bromo-deoxyuridine
c^{7}A, c^{7}I	7-deaza-adenine and -inosine derivatives
C.D.	Circular dichroism
Con A	Concanavalin A
Cyd	Cytidine
dADP	Deoxyadenosine diphosphate
d(A-T)$_n$	Alternating copolymer of polydeoxyadenylic and polythymidylic acids
DCCD	Dicyclohexylcarbodiimide
DEAE	Diethylaminoethyl
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
ds	Double stranded
DTT(DTE)	Dithiothreitol (-erythritol)
EDTA	Ethylenediaminetetraacetic acid
Guo	Guanosine
HFF	Human foreskin fibroblast
I.C, etc.	Base pairs
I_n, A_n, etc.	Homopolyribonucleotides
$I_n \cdot C_n$, etc.	Base paired hybrid between polyribonucleotides
Ino	Inosine
IF	Interferon
K_I	Inhibition constant
mRNA	Messenger ribonucleic acid
n^{8}AMP	8-amino adenosine monophosphate
NDP	Nucleoside diphosphate
NDV	Newcastle disease virus

NMP	Nucleoside monophosphate
NTP	Nucleoside triphosphate
PBS	Phosphate-buffered saline
PNPase	Polynucleotide phosphorylase
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
SE	Sulphoethyl
SFM	Serum-free medium
ss	Single stranded
TCA	Trichloroacetic acid
tlc	Thin layer chromatography
T_m	Melting temperature
Urd	Uridine
X	Xanthosine
X_N	Mole fraction of nucleotide
z^8AMP	8-azido adenosine monophosphate
$(z^8I, I)_n$, etc.	Copolymer composed of 8-azido inosine and inosine, etc.

SUMMARY

The use of affinity labelling to obtain details of interactions occurring amongst macromolecules was investigated by way of the polynucleotide phosphorylase catalysed synthesis of co-polymeric photoaffinity labels consisting of 8-azido purine and unsubstituted purine ribonucleotides. No homopolymerisation of the 8-azido-adenosine and-inosine diphosphate could be achieved even under forcing conditions. The co-polymers were characterised in some detail and it was concluded that while low levels of 8-azido substitution affected the overall stability of the single strands by virtue of a predominantly syn conformation, the degree of destabilisation was not sufficient to alter the stacking and base pairing interactions commonly encountered in the homopolymers. At high levels of substitution such destructive effects were observed, however.

During this stage of the research, a novel reaction between 8-azido-adenosine (and inosine) derivatives and dithiols was observed. Rapid reduction of the azide to amine occurred with concomitant oxidation of the dithiol. The high rate of reaction at moderately alkaline pH values and with certain dithiols, together with the virtual absence of reaction of monothiols under similar conditions suggested a ring-closure mechanism to form cyclic disulphides as a likely pathway for this important reaction.

Studies in both ribonuclease A and E. coli RNA polymerase were pursued with the photolabels. Complex patterns of labelling were generated with ribonuclease whereas an unequivocal demonstration of the β' and β subunits of RNA polymerase as the major components of the nucleic acid binding site was achieved, showing the attraction of this particular photoaffinity approach to be valid.

In the interferon system it was hoped to identify, or at least disclose the likelihood of, surface receptors important as mediators of double stranded RNA interferon induction. The 8-azido-inosine co-polymer complex with polycytidylate was an inducer of interferon, but the attempted photolabelling was hampered by the existence of a light-induced superinduction of interferon at the wavelengths necessary for photolability. Subsequently, quite strong evidence was obtained that interferon induction by synthetic inducers is related to uptake of RNA by the cell, since treatments normally found to suppress interferon production in other cell lines by presumed removal of surface bound RNA were not effective in the high-yielding cell line MG63. Furthermore, these cells were capable of pinocytosing macromolecular species at low temperatures.

CHAPTER ONE

1.1 Chemical Modification as a Probe of Biological Interactions

Since the discovery of the secondary structure of DNA, it has been apparent that such conformational knowledge of a macromolecule might provide major clues concerning the function of that macromolecule in a given system. Thus, there has followed a period of intense activity when the development of techniques for sequencing of macromolecules in terms of their monomeric units has been developed. This was particularly important for proteins when it was appreciated that the primary sequence of the peptide chain profoundly affected the secondary and tertiary structure of that protein. For the case of nucleic acids, the sequence complexity of the four major bases had much less obvious effects on the secondary and tertiary structure of the macromolecule, at least for duplex species, and only relatively recently has the importance of sequence-function relationships in terms of recognition processes at e.g. promoter -operator sites, been appreciated.

At the same time the development of techniques of high resolution X-ray crystallography allowed details of the three-dimensional structure of these macromolecules to be directly observed, and attempted correlations between the primary structure of a polypeptide and its secondary and tertiary structure are now commonplace. While this approach has only been moderately successful so far, the knowledge of the overall structure of a macromolecule has allowed speculation concerning the nature of ligand-macromolecule interactions, and contributes toward an understanding of the relationship between structure and function in such molecules. Thus, while certain structural features and chemical groupings could be inferred to be important in a given situation, the actual proof of their importance must necessarily come from direct experimental observation on the system in question. Hence, data from an X-ray diffraction pattern will only give a static picture of the macromolecule, and even though it has proved possible in some cases to crystallise proteins with their ligands still bound, it is still necessary to investigate the system in solution, i.e. in the normal dynamic situation.

For an unambiguous delineation of the relationship between ligand and macromolecular receptor, a conformational analysis of the system in the functioning state is required. In recent years it has proved increasingly possible to utilise techniques that were once only to be found in the domain of the physical chemist. Thus, much precise structural information is now obtainable from such techniques as nuclear magnetic resonance, electron spin resonance, circular dichroism and neutron scattering on biological systems present in aqueous solution (e.g. Wüthrich, 1976). However, as such a system is usually composed of many hundreds of atoms in different chemical environments, the interpretation of the data generated can be extremely difficult. The structural basis of important interactions within a given system, as deduced from such data, is still at an early stage of development, but will undoubtedly become more refined as higher resolution instruments begin to discriminate in areas of highly complex spectra.

Between the established static approach to such problems and the newly-developing dynamic spectroscopic investigations is a basically simpler area where a vast amount of research has taken place. The identification, by chemical means, of regions in macromolecules important for recognition processes, catalytic processes, co-operative and conformational changes and other binding phenomena provides the necessary bridge between the contrasting approaches of the preceding paragraphs. Information gained by chemical means has correlated well in many cases with the data obtained by crystallographers, and by using well-documented systems the new spectroscopic data can be interpreted more easily, thereby establishing a set of ground-rules for application to more intricate systems.

The earliest work on a chemical approach to closer identification of ligand-macromolecule interactions was based on studies of enzymes and their substrates. Inherent in this type of study was the identification of those residues in the active site of an enzyme that were important

to the catalytic activity of the protein (Singer, 1967). With the development of more advanced methods, complex interaction phenomena can be, and have been, tackled with confidence, and studies on a variety of macromolecular systems, e.g. antigen-antibody, effector-receptor, protein-nucleic acid, ribosomal interactions and membrane transport systems, are all areas of current acitivity.

The crudest form of investigation makes use of chemical reagents that will preferentially react with one type of amino acid residue, i.e. a group-specific reagent. When those residues that are important for the catalytic activity of the enzyme have been selectively blocked by the reagent, then the enzyme will no longer be active towards its natural substrates. By making use of radioactively labelled reagents, it should be possible to degrade the inactive enzyme and detect the amino acid residue so labelled. Once detected as an essential amino acid for catalytic activity, a certain amount of speculation over the mechanism of catalysis can be attempted. (For a review of a broad range of group-specific reagents, see Glazer (1976)). In an idealised situation, the group-specific reagent will react with 1:1 stoichiometry with the enzyme in question, concomitant with a complete loss of activity: such situations have been realised, but these cases are the exception rather than the rule, since normally only enzymes that have a micro-environment providing conditions in which amino acid side chains become uniquely reactive will react in this way. As examples of the successful application of this type may be cited the protease papain, which contains a uniquely reactive thiol group that is preferentially labelled by iodoacetate (Light et al., 1964), whilst the ε-NH_2 group of lys-41 in ribonuclease A is selectively arylated by 2,4-fluorodinitrobenzene (Hirs et al., 1966; see Figure 1.1). Even in cases of 1:1 stoichiometry, it is possible that the group-specific reagent has labelled outside the substrate binding site, causing steric impedance to approach of the substrate, or else has triggered a conformational change from outside the active site leading to an inactive form of the enzyme. A more common observation

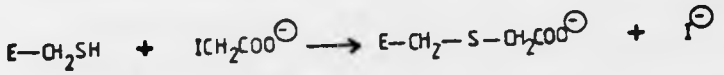
Figure 1.1

Iodoacetate and 2, 4-fluorodinitrobenzene as examples of group-specific reagents

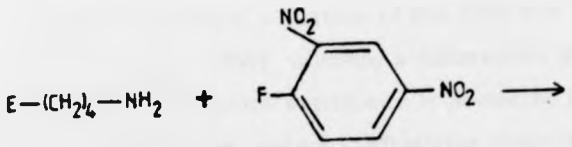
Figure 1.2

Inactivation of chymotrypsin by the quasi-substrate phenylmethylsulphonyl fluoride (Gold, 1965)

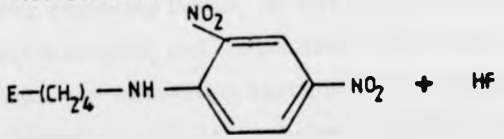
1.1



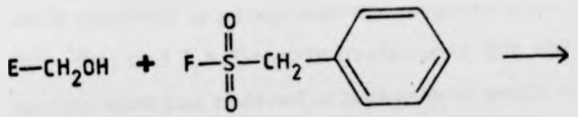
iodoacetate



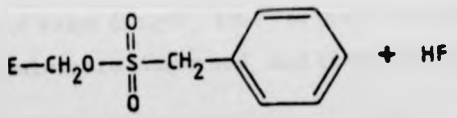
2,4-fluorodinitrobenzene



1.2



phenylmethylsulphonyl fluoride



in studies of this kind is a gradual time-dependent loss of enzyme activity, correlating with a larger number of reagent molecules bound per macromolecule. In this case, it is likely that both "exposed" and "buried" amino acid side chains are being modified, and the problems of identification of which amino acids are important functionally becomes more difficult.

A kinetic analysis of the inhibition of the enzyme or the rate of reaction of the group-specific reagent can provide important clues as to the different reactivities of the various amino acids modified (Birkett et al., 1970), and stopping the reaction at an appropriate time will often allow the most reactive residue to be identified. A valuable approach in situations of this kind was first proposed by Koshland et al. (1959), whereby a differential labelling technique is employed. Here the active site is protected by a natural ligand or ligand-analogue, and non-radioactive reagent allowed to react with the enzyme. After removing ligand, the site protected can be labelled with radioactive reagent, and the position of the residue subsequently identified. An interesting example of the technique was provided by Coombs et al. (1964) who studied the modification of carboxypeptidase A in both the native and apoenzyme forms. In the absence of the essential zinc atom, both the α-amino group of the N-terminal asparagine and the thiol group of the single cysteine were modified by group-specific reagents such as p-mercuribenzoate (for -SH) and 2, 4-fluorodinitrobenzene (for $-NH_2$). The degree of modification was reduced to background levels when Zn was added to the enzyme, implying that both groups modified are ligands of the essential Zn atom in the native enzyme. The differential labelling approach has proved of value (Singer, 1967) in many instances, both where group specific labels are concerned, and in affinity labelling studies (see below).

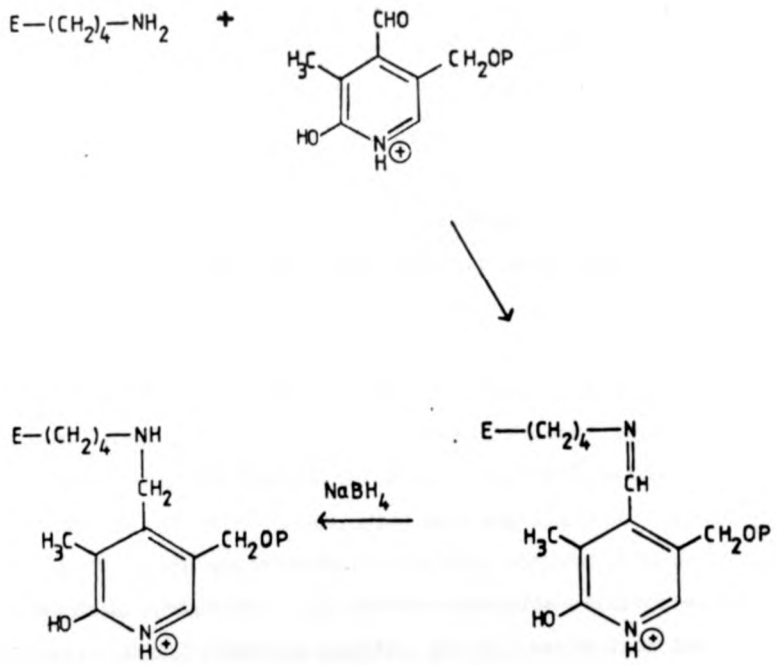
It has often proved possible to label active sites selectively by virtue of the abortive catalytic action of the enzyme itself. In this approach, advantage is taken of the high reactivity displayed by some

active site residue side-chains towards catalytically inert, but substrate-like reagents (Koshland, 1960). These quasi-(or pseudo)-substrates possess structural features that enable the active site residue (usually a nucleophile) to engage in covalent bond formation in analogy to the putative first stage of substrate catalysis. The covalent bond is, however, a stable one due to the design of the reagent, in contrast to the normal substrate linkage, and hence the residue involved can be easily identified by use of radioactive reagents. The classical reagents in this area are dialkylfluorophosphates and aryl sulphonyl fluorides which rapidly and quantitatively inactivate certain proteases by formation of a covalent bond with the active site serine residue and loss of fluoride ion (see Figure 1.2). The reagent itself need not bear any three-dimensional resemblance to the normal substrate, only a moiety capable of entering into the correct orientation for reaction to occur is required. An ideal approach to active site labelling, though of rather limited application, is to use the natural substrate of the enzyme in question; such an apparent anomaly has been made possible by a drastic alteration of conditions, e.g. temperature-drop, pH change or chemical freezing of intermediates while catalysis is proceeding. A common example of the latter type involves the reduction by sodium borohydride of the Schiff base intermediates that are formed in many catalyses where amino groups attack the unsaturated carbonyl moiety of a substrate or cofactor, e.g. pyridoxal phosphate in glutamate decarboxylase (Strausbach and Fischer, 1970; see Figure 1.3). Isolation of the active site residue under conditions in which the covalent bond formed is maintained in a stable form is then performed.

In a different approach, a selective reagent can be designed, based on close structural analogies to the natural ligand, but possessing additional reactive groupings able to interact covalently with the enzyme. Such a reagent should be highly selective by virtue of the same features that make a natural substrate bind tightly to the active site, i.e. the correct conformational parameters to enable a highly

Figure 1.3

Modification of lysyl residues by reduction of a Schiff base formed with pyridoxal 5'-phosphate


E—(CH2)4—NH2
CHO
H3C
CH2OP
HO
N
H
E—(CH2)4—N
CH
E—(CH2)4—NH
CH2
NaBH4

specific but reversible interaction to take place. These reagents are known as active-site-directed irreversible inhibitors (Baker 1967), but are more commonly known as affinity labels. It will be appreciated that the success of the affinity labelling approach depends on the availability of suitable amino acid residues at or near the active site for reaction with the active group. These amino acid residues may be important to the actual catalytic mechanism itself, but it is also quite possible that they may be important only in promoting correct binding between the enzyme and its natural substrate, i.e. a contact residue. The adaptability of the technique is shown in situations where no covalent reaction takes place, because the labelling group is not in a correct orientation to react with a neighbouring amino acid when present in the active site. Alteration of the siting of the reactive moiety within the affinity label can often produce an effective labelling species. Of the three reagents used for affinity labelling of chymotrypsin shown in Figure 1.4, I irreversibly labels histidine-57, II forms a covalent bond with methionine-192 and III (an L-analogue) does not inactivate the enzyme at all. Inactivation takes place by displacement of halogen by nucleophilic side chains. All three are based on a stereochemical similarity to natural substrate peptides, but the results show the specificity that can be achieved by suitable adjustment of the structures.

Early work with affinity labels (Schoellmann and Shaw, 1962; Wofsy et al., 1962) opened up the field to an extent that selective criteria for effective proof of affinity labelling are now apparent. Thus it needs to be shown that:

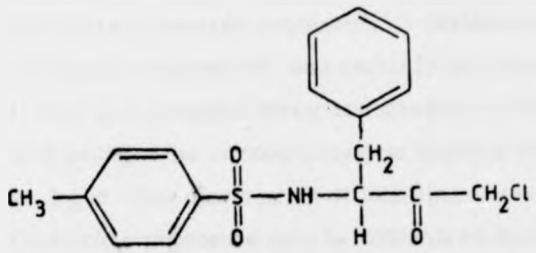
(a) the label is either a substrate for the enzyme or extremely substrate-like;

(b) that labelling is stoichiometric and can be reduced in proportion to its binding constant by labelling in the presence of natural substrate.

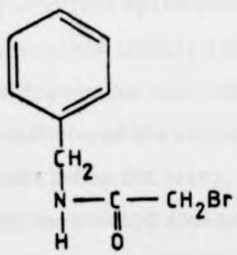
If the label is a substrate analogue, then the interaction with the enzyme will follow Michaelis-Menten kinetics, and hence the rate of

Figure 1.4

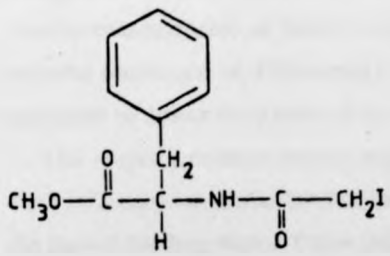
Some affinity labels used in the study of the active site of chymotrypsin



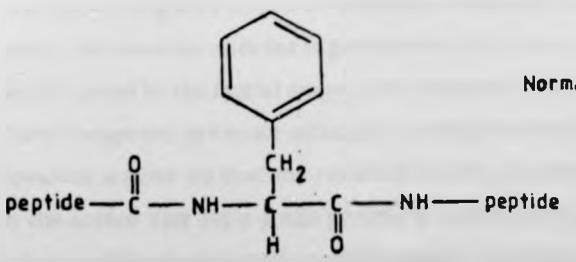
I



II



III



Normal chymotrypsin substrate

inactivation with change in reagent concentration should reach a plateau. Such considerations are necessary but not sufficient conditions for affinity labelling, although taken together they constitute a powerful argument. A further criterion, known as "catalytic competence" was recently put forward by Groman et al., (1975) who proposed that it is necessary to show that the enzyme can still perform its correct catalytic function on the bound substrate analogue for a direct proof of labelling at the substrate site. Catalytic competence may be difficult to demonstrate, as important residues for the catalytic action of the enzyme may well have been covalently modified by the putative affinity label.

The approach to affinity labelling as outlined is, however, not without its drawbacks and indeed suffers from some of the problems of non-specificity of the simpler methods. Thus, since the reactive group is carried by the label, it can react with any suitably susceptible amino acid side chain on the surface of the macromolecule. Such a reaction at an allosteric site might well cause conformational changes leading to inactivity. Performing the experiments at very low concentration ratios of label to macromolecule (below K_m) together with the technique of differential labelling discussed above, can be expected to lower the extent of non-specific labelling.

The major problem in this approach then is to be certain that such labelling as is obtained arises from interaction at, and only at, the ligand binding site. Some recent attempts at a more defined type of affinity labelling have come closer to approaching this ideal. In the first type, reagents known as suicide substrates are used. In this case, the reactive species is generated only after the reagent has participated in the initial stages of catalysis (Abeles and Maycock, 1976). These reagents are most effective when the mode of catalysis is by covalent means so that the reactive centre generated is actually held in the active site for a finite period in order that it may react with a neighbouring amino acid residue before breakdown of the reversible

enzyme-substrate complex occurs. Such affinity labels are conceptually most attractive, and many have been described (Abeles and Maycock, 1976). A simple example for plasma amine oxidase is shown in Figure 1.5. Indeed, many naturally occurring enzyme inhibitors, e.g. small molecule toxins, exhibit their mode of action by acting as suicide substrates (Rando, 1975).

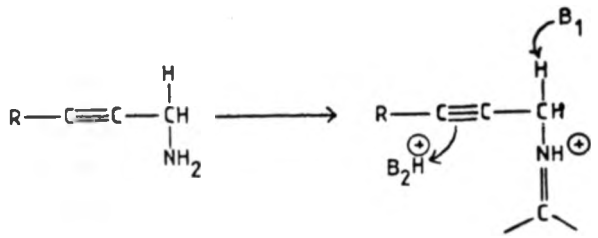
Transition state analogues are extremely powerful reversible enzyme inhibitors, and these reagents should be the most specific affinity labels yet produced (Wolfenden, 1969). Transition-state analogues are often bound more tightly by several orders of magnitude than the natural substrates for an enzyme, and it is believed that this is because such inhibitors resemble very closely the geometry of the substrate as present in the transition state of the enzyme-catalysed reaction, where extremely powerful forces of attraction are developed. The development of transition state analogue inhibitors appears to be highly promising as this should label enzymes with a specificity previously thought unapproachable. Such a reagent has been described for β-galactosidase (Wentworth and Wolfenden, 1975; see Figure 1.6). Although the inhibition was not irreversible, it occurred with a very slow rate constant consonant with a chemical intermediate of high activation energy (i.e. akin to a transition state) being formed, and was sensitive to kinetic isotope effects in deuterium oxide. These reagents, while being superior to conventional affinity labels, are however only practicable in cases where a substrate actually undergoes a transformation by involvement in catalysis and a true transition-state intermediate would be involved.

There are many other circumstances where conventional affinity labelling techniques fall short of the ideal requirements of a site-specific reagent. Thus, many important interactive processes are known where the conventional electrophilic or nucleophilic residues of enzymes are absent. In these cases specificity of interaction and subsequent functional consequences are achieved through a combination of superior steric complementarity and highly attractive hydrophobic or

Figure 1.5

A suicide substrate for plasma amine oxidase (Abeles and Maycock, 1976).

B_1 and B_2 represent groups in the active site of the enzyme



Enzyme bound intermediate

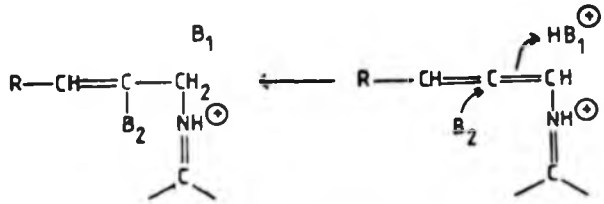
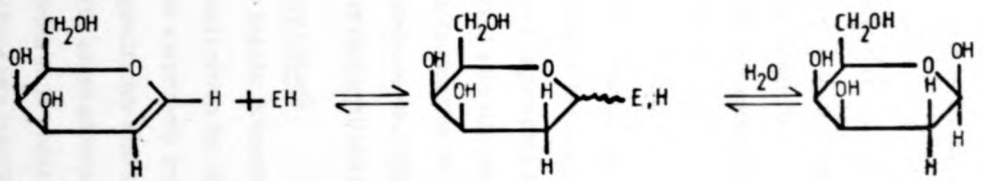


Figure 1.6

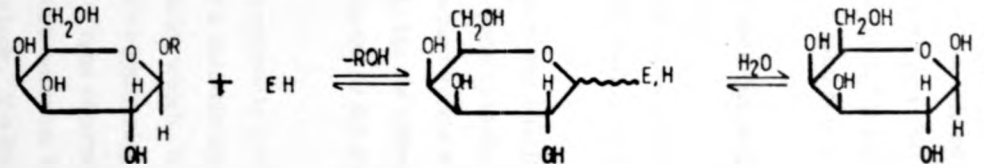
Probable mechanism of transition-state-analogue inhibition of β-galactosidase by D-galactal (Wentworth and Wolfenden, 1975)

Inhibition



D - Galactal

Catalysis



electrostatic forces. Examples of this type of interaction are receptor-effector, antibody-antigen, ribosomal and chromosomal interactions. Here the design of a substrate analogue with conventional affinity label principles incorporated is not likely to produce useful information for a number of reasons.

(a) In a complex multi-component system, e.g. a membrane, the extent of non-specific labelling will almost certainly be very high because of the large number of reactive groupings that may be available. In particular, the arrival of the ligand at the active site might well be preceded by a high degree of contact in area bordering the site of interest.

(b) Low levels of incorporation might be seen due to the absence of any suitably reactive residue in the binding site.

(c) The introduction of a bulky substituent into a small ligand might well alter its shape, perturbing complex formation by a considerable lowering of the normal binding constant.

These are general arguments which apply to any affinity labelling approach of the kind already described. Such considerations have been met by the introduction of reagents which retain all the advantages of a conventional affinity label, but the chemical reactivity of which can be veiled until the moment chosen by the investigator.

1.2 Photoaffinity Labels

Photoaffinity labels (Knowles, 1972) commonly contain a moiety which, on photoactivation by radiation of a suitable wavelength, is converted into an extremely reactive species which will ideally insert without specificity into its nearest neighbouring chemical residue. One of the chief advantages in this type of procedure is that in complex systems it is possible to ensure optimum interaction with the receptors before any labelling takes place. If a high-speed non-specific reaction subsequent to photolysis ensues, the degree of non-specific labelling will be low and the inherent information content of the technique high.

The commonly used precursors of such highly reactive species are compounds containing the diazo or azido groups which, upon photolysis, can be expected to generate carbenes and nitrenes respectively, both of which are highly reactive intermediates which insert into C-H, O-H and N-H bonds (Figure 1.7). Since the original experiments conducted by Singh et al. (1962) with a diazoacetyl derivative of chymotrypsin, there have been many papers reporting the use of photoaffinity labels. The majority, however, have used azides as their precursors. To understand why this has been so, it is instructive to consider some of the criteria necessary for a compound to be a worthwhile photoaffinity label in relation to the chemistry of azides or diazo compounds. Ideal requirements include:

(i) the label should be chemically inert in the aqueous solutions used in biological systems;

(ii) smooth photolytic conversion to reactive species should be accomplished at wavelengths clear of the absorption of the receptor system;

(iii) rearrangements of the reactive species should not occur as this is likely to lower the reactivity drastically and hence non-specificity of insertion;

(iv) ease of synthesis using stable reagents;

(v) the rate of reaction of the species generated should be extremely high so that diffusion of the reagent out of the ligand-binding site cannot occur before covalent insertion has taken place.

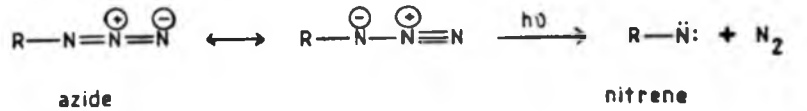
Few reagents so far designed have met all the stringent requirements The last criterion is as much a function of the affinity group as of the photogenerated species, since a ligand-analogue with an extremely high binding constant, due to good steric complementarity to the natural ligand, is less likely to diffuse out of the site and so give rise to non-specific labelling or "pseudo-photoaffinity" labelling (Ruoho et al., 1973). However, in many cases where a high binding constant has not been achieved, the use of carbene rather than nitrene precursors is much more satisfactory, due to their inherently greater reactivity (and hence non-selectivity) when photo-activated (Knowles, 1972).

Figure 1.7

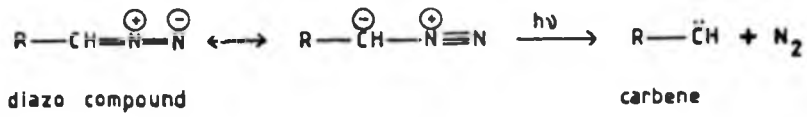
(a) Photolytic decomposition of azides and diazo compounds

(b) General reactions of nitrenes

a)



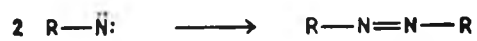
azide nitrene



diazo compound carbene

b)

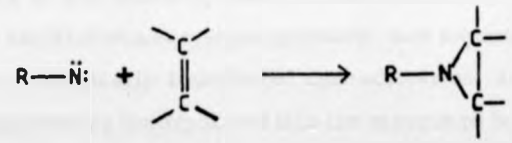
Recombination



Insertion

Abstraction

Addition



The lifetime of nitrenes is appreciably longer (Reiser et al., 1968) and their insertion reactions rather more discriminating, and in a recent study of membrane-soluble reagents it was found that the carbene precursors were much more effective on this condition alone than their strictly comparable (in binding terms) nitrene precursor counterparts (Bayley and Knowles, 1978a, b).

This advantage of carbene precursors is, unfortunately, outweighed in many cases by the problems of satisfying the first four criteria; since carbenes are very susceptible to rearrangement, their precursors are often very reactive molecules in their own right, and their absorption maxima are usually at low wavelengths where damage to receptors is likely to occur. One class of carbene precursor recently proposed are the aryldiazirines which appear to hold great promise in terms of all the chemical criteria (Smith and Knowles, 1975) and these have been successfully employed in membrane labelling experiments, as above (Bayley and Knowles, 1978b).

Most investigators have so far used nitrene precursors, i.e. azides, as the starting point in the design of their potential photoaffinity labels; in particular, it is clear that aryl nitrenes can satisfy all criteria except the caveat noted regarding the lifetime of nitrene species.

Ruoho et al. (1973) delineated some important rules for the assessment of whether a given reagent was a true photoaffinity label in the system under study. In their research on acetylcholinesterase they used a putative photoaffinity label that inactivated the enzyme completely on irradiation. However, when the active site was protected by a suitable substrate analogue, the amount of radioactivity incorporated after irradiation was undiminished, showing that the photoaffinity label was in fact labelling non-specifically over the surface of the enzyme. The addition of a scavenger molecule was expected to act as a sink for non-specifically bound label upon activation, and hence reduce the radioactivity incorporated into the enzyme to background levels. However, a complex result was obtained, since not

only was the radioactivity incorporated reduced, but the enzyme remained active after irradiation. It would appear that the scavenger, while effectively reducing non-specific reaction, was also a very efficient protector of the active site. The conclusion to be drawn from these results is that of the rate of diffusion of the activated label out of the active site is greater than the speed with which covalent insertion at the active site can occur, then one is following a situation of "pseudo" photoaffinity labelling, which is analogous in every sense to ordinary affinity labelling. The use of scavenger molecules is likely to be of value where the binding constant is not as high as desired, but it should be clear that such scavengers do not interact with the macromolecule under investigation.

Because of the potential reactivity of the reagents in terms of random labelling, it has become necessary to control very carefully for a genuine site-specific and UV-dependent incorporation. Thus it must be shown that no incorporation or loss of activity occurs in the absence of UV or when using pre-irradiated label. The photolysis treatment must not damage the macromolecular receptor, and any protection afforded by substrate analogues must not be due to their self-absorption of light, thereby precluding the photolytic destruction of the label itself. On prolonged photolysis of a ligand-protected system, there will still be a time-dependent inactivation of the receptor, since the physical interactions being dealt with are reversible. Hence, at any time there is constant diffusion of ligand into and out of the active site, and as long as any label remains unphotolysed, inactivation can continue slowly. The reversible nature of the interaction (before photolysis) is one of the great strengths of the technique, since it is possible to quantitate the affinity of the receptor for ligand, and thus to make a prior assessment of the likely specificity of interaction. The kinetics of inhibition by the unphotolysed reagent are saturable, as for a normal competitive inhibitor, and hence when present at a concentration in the region of K_I, the

inactivation of the receptor will be approximately half-maximal. The fact that the concentration of label can be manipulated prior to activation is of great advantage, since specificity of labelling can be increased still further by working at a low concentration of reagent relative to K_i.

Thus, when all criteria have been satisfied, the technique of photoaffinity labelling would appear to offer peculiar advantages in studying particular systems. For the straightforward mapping of specific residues found at the active sites, however, there are a number of diadvantages (e.g. Hixson and Hixson, 1975; Richards et al., 1974). By virtue of the non-specific insertion reactions, the number of products generated in the binding site may be several, arising from different modes of insertion into one amino acid, and the probability that more than one amino acid will be labelled anyway due to rotational motion of the label prior to reaction. The analysis of, for example an enzyme active site so labelled may well be fairly complicated, given that modified peptides might be difficult to detect in normal fingerprinting systems, and amino acids may be modified in several different ways making investigation all the more difficult (for examples see Section 3.1.1). However, it appears to hold much more promise in the field of identifying ligand binding sites within macromolecules and more complex arrays, as well as identifying sites of action of both small and large ligands, e.g. hormones or polynucleotides in multi-component systems such as organelles or in cellular tissue itself.

Aside from considerations of ligand binding, fresh opportunities are presented for the study of the three-dimensional organisation of macromolecules in terms of the disposition of such molecules with regard to each other, and by extension, the arrangement of subunits within a given macromolecule. Thus a number of small molecule photolabile cross-linking reagents has been described which appear to be more useful than the standard type of bifunctional reagent

(Davies and Stark, 1970) which relies on the fortuitous proximity of reactive residues on the surface of the macromolecules. The photolabile cross-linkers are heterobifunctional in that they contain a moiety capable of reacting with nucleophilic groups on proteins, e.g. halo-acyl or imidate functions, together with a photo-activatable group which will insert non-specifically with a contact residue upon subsequent photolysis. To date such reagents have been used with some success in diverse studies as membrane receptor identification (Ji, 1977), spatial disposition of residues in active sites (Hixson and Hixson, 1975) and identification of cytochrome proximities in the electron transport chain (Ereciński et al., 1975, Ereciński, 1977). The utility of the approach seems likely to be general and indeed appears to be an extremely effective procedure for generating data on inter- and intra-molecular organization in multi-component systems.

By far the largest portion of research in affinity labelling has concentrated on small molecule probes, naturally enough, for it is in this area that both the synthetic aspect and the interpretation of results are at their (relatively) most simple. In recent years, however, it has become apparent that the complex macromolecular interactions that underline the processes of replication, transcription and translation, as well as forming the basis of cellular structures, are amenable to study both by physical and chemical techniques. While the dramatic increase in our knowledge of the structure of chromatin has been brought about in large part by an application of spectroscopic techniques (Felsenfeld, 1978), the complex ribosomal protein synthetic system has been investigated more successfully by the use of affinity labelling (Pellegrini and Cantor, 1977).

1.3 Macromolecular Affinity Labels

A large multi-component complex, such as the ribosome, can offer advantages when utilising large, as opposed to small, molecule affinity labels. It is possible to effect a substantial modification to the

macromolecule, in terms of the introduction of a suitable reactive group, without affecting the overall binding specificity or dissociation constant. This will be the case since a large number of non-bonded contacts are usually present, leading to the observed tight binding. This same feature can be taken advantage of, enabling low concentrations of affinity label to be used, with concomitant improvement in the level of non-specific side reactions. However, where only a low level of labelling is observed, it is important to show that the system was initially functionally intact for the results to be of relevance. If a few important proteins were missing from a percentage of ribosomes in a given preparation, then a valid argument against the importance of a putative interaction would be that labelling was only occurring on depleted ribosomes, sufficient of which were present to account for the data. Thus the importance of meaningful controls is paramount to the interpretation of a given experiment. At the very least it must be shown that the requirements for ligand binding are the same in terms of buffer and/or cofactor concentrations and that the analogue is a competitive inhibitor of natural ligand binding. The best criterion on which to proceed, however, is the demonstration that the analogue can actually substitute for the natural ligand in terms of the functional consequences of its binding and in the transformation that it actually undergoes.

The interpretation of the labelling patterns of necessity are likely to be difficult since one will be trying to identify a recognisable component attached to the macromolecular affinity label. It is necessary to destroy the majority of the ligand, by hydrolysis for example, so that only those parts of it covalently attached to the binding site of interest remain. Standard procedures of macromolecule purification and identification such as SDS gel electrophoresis or peptide mapping are employed, but the covalent modification may drastically alter the properties of the labelled species, leading

to distorted patterns. In the last analysis, the only unambiguous means is to sequence the component so modified, be it protein or nucleic acid. This will require larger quantities of material than are often available, but the bonus of identifying not only the macromolecular species of interest, but also its precise site of interaction with neighbouring macromolecules should allow descriptions of structure-function relationships in these cases to reach the level attainable with simple enzyme-substrate systems.

1.4 Aims of the Present Investigation

Despite the enormous use of affinity labelling to investigate small ligand-protein interactions in vitro, little research has been carried out on corresponding nucleic acid-protein interactions, possibly due to synthetic problems of introducing suitably reactive groups into the nucleic acid. An exception here is tRNA, which has been modified both at the 3'-end and at the 4-thiouridine base. The introduction of an electrophile into a nucleic acid to form a potentially large number of reactive sites may lead to difficulties in obtaining true equilibration with a given protein, owing to rapid non-specific reactions.

On the other hand, the promising results recently obtained by use of photoaffinity labelling provides grounds for believing that the development of suitable model systems in the area of nucleic acid interactions with their receptors would be of finite value and importance.

Using the enzyme polynucleotide phosphorylase, we chose to investigate the synthesis of polynucleotides containing photolabile groups, thereby producing a series of RNA analogues to be used in systems that commonly utilise homoribopolynucleotides as substrates or as ligands.

Identification of the necessary conditions for synthesis of such polynucleotides would necessarily be followed by a detailed characterisation of the polymers in terms of their physical properties, in order to assess their likely usefulness in a variety of systems (see Chapter Two). In particular, the study of the complex formation with

complementary polyribonucleotides was an important preliminary to the decision to initiate an investigation into the phenomenon of interferon induction (see below).

Once the polynucleotides had been characterised, they were tested for their ability to act as photoaffinity labels in two enzymic systems, viz. pancreatic ribonuclease and E. coli RNA polymerase. Both enzymes utilise polyribonucleotides as substrates, so we searched for the most efficient conditions for specific photoaffinity labelling. It was also hoped that novel structural information regarding the nature of the respective polynucleotide binding sites of these enzymes would be found. The results of these investigations are reported in Chapter Three.

The use of affinity labels to investigate interactions between macromolecules and their putative receptors on cells in culture is a very undefined area. A particularly interesting system, over which the data for the identity of receptors (or whether in fact they exist) is still much confused, is the induction of the anti-viral substance(s), collectively known as interferons. In the present study, it was the intention to prepare a photoaffinity label capable of eliciting a comparable interferon response to other synthetic inducers when administered to cells. In so doing, we hoped to glean information as to whether induction from the cellular membranes (and thus presumably via binding to a receptor) was a probable mode of action of polyribonucleotide inducers. The question of penetration of synthetic inducers of interferon has been posed and remained unanswered for some years. The results of this section of the work appear in Chapter Four.

In the course of this study, two other experimental observations, not entirely connected with the main theme of the work as outlined above, were pursued in some depth because of the important light they shed

(a) on the use of photoaffinity labelling under certain commonly encountered conditions;

(b) on the induction of interferon at the membrane level.

CHAPTER TWO

2.1 Introduction

2.1.1 Azido Nucleotides

The earliest report concerning the introduction of the azido group (z) into a nucleoside base was by Holmes and Robins (1965) who utilised a reaction involving nucleophilic displacement of bromide from 8-bromo-adenosine (br^8Ado) by the azide moiety derived from sodium azide in dimethylformamide. It was later found that the same reactions could be applied to the nucleotide cAMP (Muneyama et al., 1971) although the route to the 8-bromo-substituted adenosine derivative was somewhat different from that used before because of the altered solubility properties of the nucleotide (Ikehara and Uesugi, 1969). That such compounds might be useful as photoaffinity labelling reagents was not generally realised until after the timely review by Knowles (1972) which set forth the advantages and possibilities of a technique which had, at that stage, been used in only a handful of published papers. It has since become apparent that photoaffinity labels based on azido nucleotides can give important structural information in many systems in which nucleotides play a role (see Chapter 3).

At the time when the project described in this thesis was initiated, there had been only one report of the use of an azido nucleotide (z^8ATP) in a photoaffinity labelling experiment (Haley and Hoffman, 1974) in which the cation-stimulated adenosine triphosphatase of red cell membranes was effectively labelled. A wide range of nucleoside and nucleotide analogues capable of deployment as photoaffinity labels has been described more recently, but of these, direct substitution in the heterocyclic base by the azido group has only been noted for purines, e.g. z^8Guo nucleotides (Geahlen and Haley, 1977), z^2Ino nucleotides (Wiegand and Kaleja, 1976) and z^8Ado nucleotides (Haley and Hoffman, 1974, and others) including NAD^+ and FAD derivatives (Koberstein, 1976).

It is noteworthy that the z^2Ino nucleotides are highly fluorescent due to the formation of a tetrazole by attack at the electrophilic N-3 of the purine ring (Fig. 2.1a). The infra-red spectra of this series show that the free azide exists at a level below 5%, thus reducing their effectiveness as suitable Ino photoaffinity analogues. In the tetrazole form, no absorption in the infra-red spectrum at the region characteristic of free azido moiety (i.e. 2100-2200 cm^{-1}) is seen (Temple et al., 1966). In N1-methyl substituted z^2Ino nucleotides, no IR absorption is seen in this region, and photolytic reactions were not detected (Wiegand and Kaleja, 1976). There have been no reports of fluorescent properties of z^8Ado nucleotides, and photolysis appears to occur readily.

Attempts to produce the corresponding free azido analogues of the pyrimidine nucleosides and nucleotides has met with continued failure (Bradshaw and Hutchinson, 1977). Although the 5-bromo pyrimidine nucleosides and nucleotides are stable and freely available compounds, direct nucleophilic attack by a variety of nucleophiles, e.g. fluoride, methoxide, does not occur. Azide ion attack leads to mixtures of products in contradiction to that expected from the comparable purine case. The reaction has been investigated in some detail by Sasaki et al. (1976), and the major products of the reaction have been isolated (Fig. 2.1b). It is apparent from this work that these 5-bromo pyrimidine bases are unexpectedly inert toward nucleophilic substitution by azide ion, and that the reaction actually proceeds by way of a 5'-azido-5'-deoxy intermediate with subsequent attack by 5'-azido on the 6-position of the base and cyclisation. In particular, it is found that if the 5' position of the ribose contains a bad leaving group, e.g. O-trityl, then the 5-bromo substituent is not replaced even after several hours reaction at high temperature.

In the work to be described below, z^8Ado nucleotides have been synthesised in reactions similar to those described initially (Muneyama et al., 1971), but with substantial modifications in

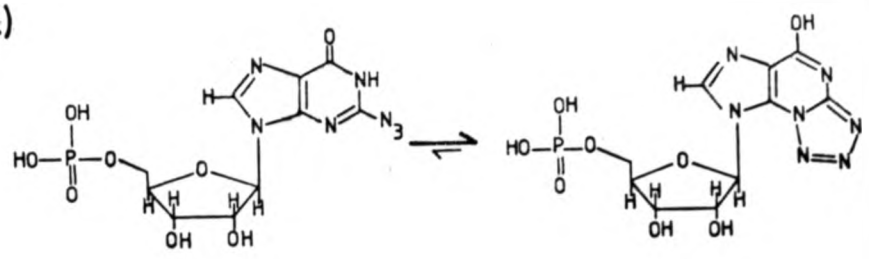
Figure 2.1

Structures of some azido substituted nucleosides and nucleotides

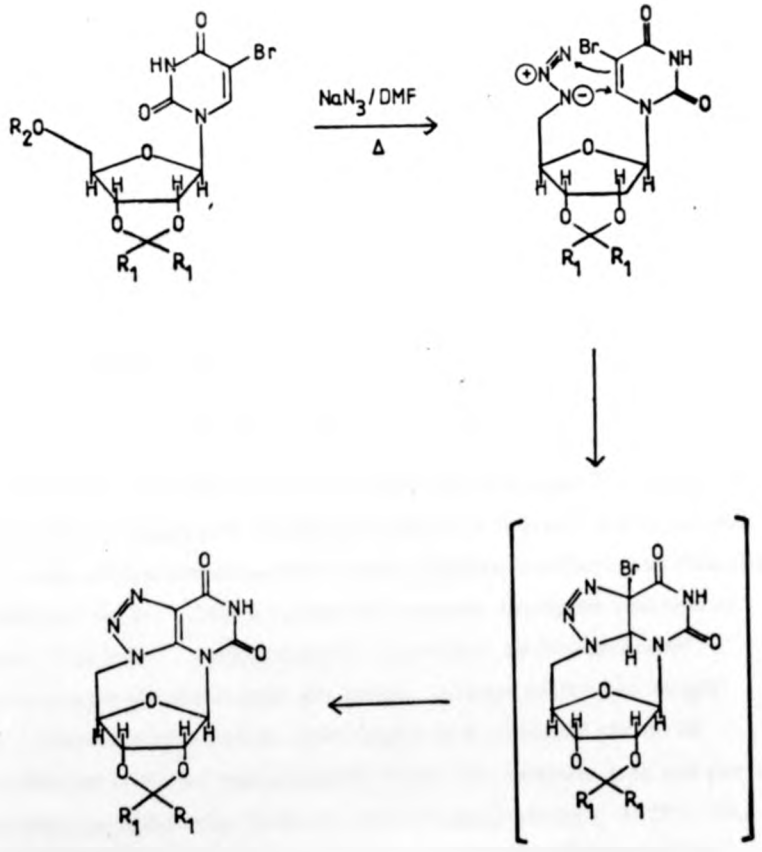
(a) 2-azido inosine monophosphate and its corresponding tetrazole (Wiegand and Kaleja, 1976)

(b) Reaction pathway for azidolysis of 5-bromo uridine (Sasaki et al., 1976)

a)



b)



R_1 = Methyl R_2 = Benzyl or Tosyl

conditions and purification procedures. z^8Ino nucleotides are synthesised from the z^8Ado nucleotides in high yield, and are described for the first time (although the cyclic monophosphate was reported earlier (Miller et al., 1973)). Both series of nucleotides have been examined for their ability to be photolysed under mild conditions.

2.1.2 Polyribonucleotide Synthesis

The discovery of polynucleotide phosphorylase (E.C.2.7.7.8) (Grunberg-Manago and Ochoa, 1955) was followed by a period of intense activity involving the synthesis of novel polyribonucleotides. The enzyme was the first nucleic acid synthesising component to be uncovered in the cell, but has proved to be one of the most intractable as far as delineating its rôle in vivo is concerned. The reaction catalysed is the condensation of ribonucleoside diphosphates to form 3'-5' linked polyribonucleotides with liberation of inorganic phosphate. The reverse reaction is also catalysed efficiently and herein lies the paradoxical nature of the enzyme, viz. is it an enzyme of nucleic acid synthesis or degradation in vivo ? With a requirement for a divalent metal cation, the reaction can be drawn schematically as:

$$n(rNDP) \xrightleftharpoons{M^{2+}} (rN)_n + nP_i$$

In bacterial systems (the usual source of the enzyme for synthetic purposes) it is present in the cytoplasm and is fairly easily purified by standard fractionation techniques (Godefroy-Colburn and Grunberg-Manago, 1972). The pH range for enzyme-catalysed reaction is from 7 to 10.5. Polymerisation appears to be favoured over phosphorolysis at the upper pH values and high molecular weight polyribonucleotide can be synthesised by a judicious choice of conditions involving temperature, metal ion concentration and period of reaction (Godefroy-Colburn and Grunberg-Manago, 1972). The enzyme shows no template requirement and will polymerise the naturally occurring nucleotides CDP, UDP, IDP and ADP to the

corresponding homopolyribonucleotides in high yield (Grunberg-Manago et al., 1956; Littauer and Kornberg, 1957) under conditions in which, however, GDP is very difficult to polymerise (see below). It does, however, often display a marked primer dependency; this is more noticeable with highly purified preparations and is markedly dependent on external factors such as divalent metal ion concentration and pH as well as partial proteolysis of the enzyme (Godefroy et al., 1970). These oligo- or polynucleotide primers are incorporated into high molecular weight product, providing a demonstration of the processive nature of polymerisation (Thang et al., 1970). Copolymerisation of a mixture of nucleoside diphosphates has also been shown to occur readily. Incorporation has been assumed to be random, and in the case of copoly (A, U) the base ratio in the product is equivalent to the input ratio of nucleotides (Ortiz and Ochoa, 1959). This latter result is not true for all copolymers (Wang and Kallenbach, 1971), a demonstration that a form of specificity resides with the enzyme-catalysed reaction. The totally random nature of the PNPase reaction is now also subject to some doubt and is thought to be solvent-dependent (Rottman and Johnson, 1969; Greene et al., 1978).

Such specificity has been particularly noticeable when the synthesis of G_n from GDP was attempted. Low yields were obtained under normal conditions (Singer et al., 1960) and it was thought possible that the enzyme was particularly sensitive to the multi-stranded structure of G_n by virtue of the equilibrium that existed between the mononucleotide and the polynucleotide in the presence of enzyme (Grunberg-Manago, 1959). The specificity requirements of the enzyme were found to be relaxed by a substitution of other divalent metal ions for Mg^{2+}; in particular, Mn^{2+} shows a marked effect (Babinet et al., 1965; Chou and Singer, 1971). Thus G_n was synthesised by the E. coli enzyme in good yield under high temperature

conditions (60° C) in the presence of Mn^{2+} ions (Thang et al., 1965). It seems likely that the metal ion cofactor is an independent substrate of the enzyme, but that an altered conformation in the active site is achieved in its presence, allowing a more relaxed specificity requirement for nucleoside diphosphates (Williams et al., 1964).

This aspect of the enzymology of polynucleotide phosphorylase has been taken full advantage of in the synthesis of a very large variety of polyribonucleotides containing altered nucleotides. Studies on these analogues has allowed a more fundamental comprehension of the factors underlying the structural stability of natural nucleic acids, both in single stranded and double stranded forms. In the particular case of interferon induction, the polyribonucleotides made available by these techniques have enabled the structural requirements of an effective ds RNA interferon inducer to be quite closely defined (see Chapter 4).

A feature of the relaxed specificity of polynucleotide phosphorylase in the presence of Mn^{2+} ions has been the relative ease of polymerisation of nucleotides with modifications of, or substituents added to, the heterocyclic base, or even nucleotides composed of different heterocycles bonded as β anomers to ribose via the glycosyl bond (Michelson et al., 1967). A particular class of these, viz. those with substituents in the 6-position of the pyrimidine ring or the 8-position of the purine ring have, however, in most cases resisted attempts to synthesise their homopolymers by enzymic means. To understand the probable reasons for this, it is necessary to consider some conformational aspects of nucleosides and nucleotides.

2.1.3 Syn and Anti Conformations

It was recognised at an early stage that nucleosides have a number of conformational degrees of freedom in the ribose molety. Of great importance was the position of the heterocyclic base relative to the sugar, a position that could change by rotation about the C1'-N glycosyl bond (Donohue and Trueblood, 1960).

In Fig. 2.2, the two main orientations allowable on steric grounds are shown, both for pyrimidine and purine nucleosides. The orientations are designated *syn* and *anti*. It will be seen that the *anti* orientation implies the lowest steric interference in terms of interactions between ribose and the exocyclic groups of pyrimidine bases or the pyrimidine ring of the purines. From an examination of molecular models, Donohue and Trueblood (1960) suggested that two allowable ranges would exist at energy minima, one being centred at φ approximately -30° (*anti*) and the other at φ approximately 150° (*syn*) (see Fig. 2.2). Crystal structure analysis showed that the natural nucleotides existed in the *anti* form (Sundaralingham, 1969), but of more relevance were solution studies in which nuclear magnetic resonance (Sarma *et al.*, 1974) and circular dichroism (Follmann *et al.*, 1975) allowed the inference to be drawn that these nucleotides did indeed consist of an *anti* population.

A detailed study by Haschemeyer and Rich (1967) based on crystal structures (together with a theoretical treatment) showed that the preferred and/or allowed conformations of the heterocyclic base about the glycosyl bond were very dependent on non-bonded contacts, which in turn depended on the conformation of the ribose sugar. In monoribonucleotides, as well as RNA, the only ribose conformation found is the $C_{3'}$ endo, and under these circumstances both purine and pyrimidine nucleotides would be expected to occur in the *anti* conformation. $C_{2'}$ endo ribose would relieve a number of non-bonded contacts and the allowable range of *syn* torsional angles is high, particularly for the purine case, so that nucleotides with this ribose puckering may have a low rotational barrier between the normal *anti* and formally less usual *syn* conformation.

Reference to Fig. 2.2 will show that substitution of a bulky group such as bromide or azide at the C-8 position of purine nucleosides or the C-6 position of pyrimidine nucleosides when in the *anti* conformation

Figure 2.2

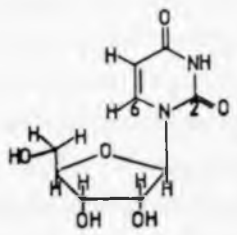
The *syn* and *anti* conformation about glycosyl bonds

(a) for pyrimidine nucleosides

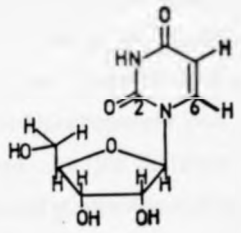
(b) for purine nucleosides

(c) depicts the Newman projection defining φ_{CN}, which is the angle between the C6-(C8)-N-C1' plane and the O1'-C1'-N plane. Positive anges are measured when C1'-O1' is rotated in a clockwise direction when viewing from C1' to N. The figure shows φ_{CN} at *ca.* -30^{o}

a)

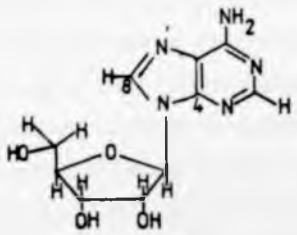


Anti

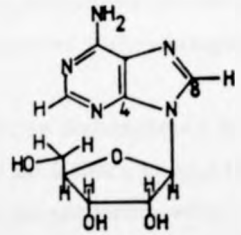


Syn

b)

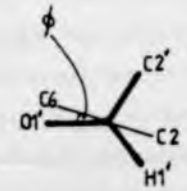


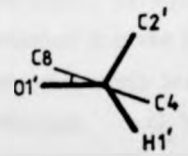
Anti



Syn

c)





is likely to produce severe steric crowding with the oxygen of the ribose ring, as well as the $C_{2'}$ and $C_{3'}$ hydrogens. The probability that such a situation could be relieved by adoption of the syn conformation was initially confirmed by crystal structure studies on the purine nucleosides br^8Guo and br^8Ado (Tavale and Sobell, 1970). Interestingly, the sugar conformation was found to be $C_{2'}$-endo as was to be expected from the studies of Haschemeyer and Rich (1970). Ikehara et al. (1972) performed circular dichroic studies on a range of 8-substituted adenosine and guanosine nucleosides and nucleotides and reported that in solution the major conformation adopted was syn. More recently, confirmation of the assignments has come from NMR studies (Sarma et al., 1974) and it is now commonplace to use CD and/or NMR techniques for accurate glycosyl conformational assessment. It would seem likely that such conformational differences in these substituted nucleotides would be reflected in altered functional properties of the analogues compared to their anti counterparts.

Polymerisation of 8-substituted Ado and Guo diphosphates by polynucleotide phosphorylase was attempted by Ikehara et al. (1969) who found essentially no homopolymer could be produced, even after long incubation times and high temperatures were employed. Qualitatively similar results were obtained by Kapuler et al. (1970) in a study of 8-substituted Guo nucleotides and 6-substituted Cyd nucleotides, even when synthesis was attempted in the presence of Mn^{2+} ion, as described above for the synthesis of G_n. In an attempt to find enzymes with the ability to produce polymers from such atypical bases, a polynucleotide phosphorylase from B. stearothermophilus was purified (Wood and Hutchinson, 1976). With a temperature optimum of 69° C, it was reasoned that the barriers to rotation of a syn nucleotide would be proportionately less and homopolymerisation might take place. Although G_n was synthesised by this enzyme smoothly and in high yield, br^8 ADP

could not be polymerised. This enzyme actually displayed a more strict specificity than that from E. coli and it was argued that this was probably a result of the inherently more rigid structure imposed on the enzyme as a prerequisite for maintenance of its integrity at these elevated temperatures. A similar PNPase from Thermus thermophilus has been described (Kikuchi et al., 1977) and this also efficiently polymerises GDP.

In an extension of the work on polynucleotide phosphorylase, Kapuler and Reich (1971) looked at the ability of the syn oriented Guo and Cyd nucleotides to act as substrates in template-directed polyribonucleotide synthesis by RNA polymerase. None of the nucleotides was utilised as substrate alone, but in analogy with the results for polynucleotide phosphorylase (Ikehara et al., 1969), it was found that low levels of incorporation into polymer (i.e. copolymer formation) were achieved in the presence of normal nucleotide substrates. In all the cases described, the syn oriented nucleotides were tightly bound inhibitors of the enzymes, indicating that the syn conformation was accommodated at the active site with the same affinity as the natural substrate, but that for phosphodiester bond formation to occur, some late stage in the catalytic process probably requires the anti conformation.

This view has been strengthened recently when it was shown that a series of 8-amino substituted ATP analogues were all competitive inhibitors of lactate dehydrogenase, and all had very comparable inhibition constants (Evans and Kaplan, 1976). Of particular interest in this study were the NMR results which showed that whilst 8-dimethyl-amino AMP was in the syn conformation, 8-amino AMP existed predominantly as the anti conformer. This was the first demonstration that an 8-substituted purine nucleotide could exist in the anti conformation in solution, and explains the ready polymerisation of n^8ADP by polynucleotide phosphorylase (Howard et al., 1972). Initially it had been thought that the $(n^8A)_n$ must be a syn polymer, but the likelihood exists that the 8-NH_2 group can hydrogen bond to

the 5'O of the ribose and hence stabilise its anti conformation. This interpretation was given more credence when it was found that 8-monomethylamino AMP also existed predominantly as an anti conformer. These NMR results have been confirmed and extended to the corresponding Guo nucleotides with the same conclusions (Jordan and Niv, 1977). That the nucleotides exist in a rapid equilibrium between two energy minima has been shown by ultrasonic relaxation studies where the barrier to syn-anti rotation was found to be approximately 6 kcal/mole for adenosine, whilst the time constant for rotation was of the order of 1 nsec (Rhodes and Schimmel, 1971). The NMR studies confirm the rapid equilibration since the line-widths observed are narrow (Evans and Kaplan, 1976). The effects of paramagnetic ions bound to the 5'-phosphate on the relaxation of protons on the purine base in 8-substituted nucleotides confirms the pattern of syn or anti conformation deduced from chemical shifts. In addition, the magnitude of such data allows a rough calculation of the relative proportions of syn and anti forms at equilibrium e.g. for 8-$N(CH_3)_2$AMP, 10% anti and 90% syn (Evans and Kaplan, 1976).

Thus it seems probable that even nucleotides which exist formally as syn conformers are not restricted to this conformation in functional terms. Abdallah et al. (1975) have shown that br^{8}ADP ribose (a syn conformer) is an inhibitor of liver alcohol dehydrogenase. X-ray analysis of a crystal of enzyme with the inhibitor in situ shows that the anti conformation is present in the complex, in analogy with that found for the natural cofactor, NAD^+. It was shown that the ribose in this crystal existed as C_2, endo, presumably in order to relieve the high steric requirement of the anti form with the normal C_3, endo. (Haschemyer and Rich, 1967).

Haley and Hoffman (1974) consider that z^{8}ATP would exist in the syn conformation, since it is an inhibitor of certain tRNA synthetases, although a substrate for myosin. The nucleotide does not substitute for ATP in template-directed RNA synthesis by E. coli RNA polymerase (V.W. Armstrong, personal communication).

However, in the light of the published work on 8-substituted purine nucleotides, it would be of interest to study the properties of z^8ADP as a substrate for polynucleotide phosphorylase, and the structural consequences of its introduction into polyribonucleotides either in the form of a homopolymer or as a copolymer. The results of this work are presented below, together with the discovery of a reaction of z^8Ado nucleotides that possesses great importance for the successful exploitation of these reagents in photoaffinity labelling experiments.

2.2 Materials and Methods

2.2.1 Materials

(a) Tetramethylguanidium azide was supplied by Lancaster Synthesis Ltd., Lancaster, U.K.

(b) Nucleotides and polynucleotides were products of Boehringer Corporation (London) Ltd.

(c) RNase A type XII-A (E.C.3.1.4.22), RNase T_1 (E.C.3.1.4.8), RNase T_2 (E.C.3.1.4.23), yeast hexokinase (E.C.2.7.1.1) and snake venom phosphodiesterase from Crotalus adamanteus (E.C. 3.1.4.1) were from Sigma (London) Chemical Co.

(d) Calf intestinal alkaline phosphatase (E.C.3.1.2.1) and M. luteus polynucleotide phosphorylase (E.C.2.7.7.8) were products of Boehringer Corporation (London) Ltd.

(e) Polynucleotide phosphorylase from E. coli and B. stearothermophilus were prepared by the method of Wood and Hutchinson (1976).

(f) Phenyl azide was the gift of Dr. G.H. Dodd, University of Warwick.

(g) $NaB[^3H]_4$ (661 mCi/mmol), α-[^{32}P]-ATP (10.3 Ci/mmol), [^{3}H]-ADP (21 Ci/mmol), [^{3}H]-AMP (22 Ci/mmol) and [^{3}H]-IDP (4 Ci/mmol) were supplied by the Radiochemical Centre, Amersham, U.K.

(h) Thin layer chromatography plates were Kieselgel 60 F_{254} and were a product of E. Merck, Darmstadt. Paper chromatography was performed on Whatman 3MM.

(i) All other reagents were of analytical grade unless otherwise stated.

2.2.2 General Methods

(a) Sedimentation values of polynucleotides were determined on a Beckman Model E analytical ultracentrifuge. The help of Dr. C.S. Dow, University of Warwick, is gratefully acknowledged.

(b) Circular dichroic spectra were obtained on a Cary 61 recording C.D. spectropolarimeter. Thanks are due to Dr. P.M. Scopes, Westfield College, University of London, for performing these.

(c) Thermal transition profiles of polynucleotides were produced on a Gilford 250 series UV spectrophotometer equipped with an automatic 2527 thermo-programmer. I am grateful to Dr. R.J. Avery, University of Warwick for the use of this instrument.

(d) Low resolution mass spectra were performed at P.C.M.U., Harwell, Berkshire, U.K.

(e) Photolysis experiments were performed with a 100 W high pressure mercury compact arc lamp (Hanovia Lamps Ltd., Slough, Bucks.). The light beam was focussed, by means of a quartz lens (20 cm focal length) on to a 2mm quartz UV cuvette containing the solution to be photolysed. Filtering of the lamp was provided by pyrex and/or soda glass filters in order to cut out the high energy wavelengths. The cuvette was kept at 2^{o} when necessary, by passing over it a stream of ice-water from a peristaltic pump.

(f) Chromatography was performed by upward development for tlc plates and downward development for paper, using the following solvent systems:

A	n-butanol:acetic acid: water	5:2:3
B	n-butanol:acetic acid: water	4:1:5 (top layer used)
C	n-butanol:water	86:14
D	iso-butyric acid: ammonia (S.G.0.88): water	66:1:33
E	iso-butyric acid:ammonia (S.G.0.88): water	57:4:39
F	1M ammonium acetate pH 7.5:ethanol	3:7
G	t-amyl alcohol:butanone:water:formic acid (S.G.1.2)	2:2:1:0.1

Spots were visualised by fluorescence under 254 nm light in the case of tlc plates. Paper chromatograms were visualised using the method of Cashion et al., (1977).

(g) Extinction coefficients of nucleotides and polynucleotides were based on phosphorus content determined by the method of Chen et al. (1956).

(h) Polynucleotide phosphorylase was assayed by polymerisation as previously described (Wood and Hutchinson, 1976). One unit of enzyme is that quantity releasing 1 μmole of inorganic phosphate per hour at 45° C.

(i) The scintillants used for counting of radioactive samples were:

(i) toluene (600 ml), 2-ethoxyethanol (400 ml), PPO (4g), POPOP (0.2g)

(ii) toluene (1000 ml), PPO (4g), POPOP (0.1 g).

(j) The kinetics of reaction between z^8Ado nucleosides (and nucleotides) and thiols was followed by the change in UV absorbance at 300 nm with time at 25° C in a variety of buffers:

(i) pH 5-7 0.1 M citrate-phosphate

(ii) pH 8-9 0.1 M borate-phosphate

(iii) pH 10-12 0.1 M glycine-sodium chloride-sodium hydroxide

Conditions used in the various experiments are given in the legends to the relevant tables and figures.

2.2.3 Synthesis of 8-bromo-adenosine monophosphate

The basic scheme of synthesis was as previously described (Ikehara and Uesugi, 1969; Cartwright, 1975), but with the introduction of a number of modifications.

To a stirred solution of potassium acetate buffer, pH 4.5 (0.5 M, 40 ml) was added adenosine 5'-monophosphate (3g, 8.2 mmole) with slight warming to aid dissolution. To this was added a solution of bromine (0.84 ml) in a potassium acetate, pH 4.5 (0.5 M, 20 ml) and stirring at room temperature was continued overnight. During this procedure, the pH dropped to 3.2 due to production of hydrogen bromide. Thin layer chromatography in solvent A showed near complete conversion of starting material. Excess bromine was dispelled by passage of nitrogen gas through the solution until the colour had changed from deep red to yellow. Reduced pressure evaporation at ca. 40° C was followed by lyophilisation of the remaining small volume. The period of evacuation was prolonged until all traces of acetic acid were removed from the residue, since any acid in the subsequent column purification step would

generate carbon dioxide gas and block the column. The red residue was dissolved in water (600 ml), applied to a column (50 x 2.5 cm) of DEAE Sephadex A25 (HCO_3^- form) and washed with triethylammonium bicarbonate, pH 8.7 (0.05 M) until all UV absorbing impurities had been eluted. Elution of br^8AMP was achieved by a linear gradient of triethylammonium bicarbonate, pH 8.7 (0.05 - 0.15 M, 2 l) applied to the column. Fractions containing the pure nucleotide (monitored by tlc as above) were pooled and evaporated to dryness under reduced pressure at low temperature. Excess triethylammonium bicarbonate was removed by repeated dissolution of the residue in methanol, and evaporation under reduced pressure, to give a pale yellow product of br^8AMP (triethylammonium salt). Yield (in terms of O.D. units) = 95%. UV data in substantial agreement with that of Ikehara and Uesugi (1969).

2.2.4 Synthesis of 8-azido AMP

All manipulations with azido compounds were performed as much as possible in the dark, i.e. reaction flasks wrapped in foil, etc.

br^8AMP (4.5 g, 7.2 mmole) was dissolved in dimethyl formamide (7.5 ml) and to the stirred solution was added as quickly as possible solid tetramethyl guanidium azide (3.4 g, 21.6 mmole, very hygroscopic compound). The solution was warmed in an oil bath to ca. 70° for approximately 30 hours, by which time tlc in solvent A and ultraviolet analysis (Holmes and Robins, 1965) showed that substantial substitution by the azido group had occurred. The DMF was distilled off under reduced pressure to leave a sticky yellow oil which was shaken with dry diethyl ether (5x) to extract as much unreacted tetramethyl guanidium azide as possible. After dissolution of the oil in water (200 ml), the solution was applied to a 50 x 2.5 cm column of DEAE Sephadex A25 (HCO_3^- form) and eluted in the same manner as for the br^8AMP purification above. Those fractions containing z^8AMP (as monitored by tlc and UV) were found to emerge slightly behind unreacted br^8AMP at approximately 0.1 M triethylammonium bicarbonate. The pooled fractions were concentrated as above to give z^8AMP in 78%

yield (in terms of O.D. units), UV (pH 7) λ_{max} 282 nm, ϵ 12,900: lit: (pH 7.4) λ_{max} 281 nm, ϵ 13,300 (Haley and Hoffmann, 1974); ν_{max} (Nujol), 2160 cm^{-1} (strong).

For some applications it was deemed necessary to have the sodium salt of z^8AMP and this was prepared readily as follows. A quantity of nucleotide was dissolved in a small volume of distilled water and applied to a column of SE Sephadex C25 (Na^+ form, 20 x 1 cm). The fractions containing nucleotide were pooled and lyophilised to give a fluffy white product of z^8AMP (sodium salt). NMR spectra of the product show all traces of triethylamine to have been eliminated by this procedure.

2.2.5 Synthesis of 8-azido ADP

The basis of the method used was that due to Moffat and Khorana (1961).

Dicyclohexylcarbodiimide was purified as follows. DCCD (5 g) was added to acetonitrile (30 ml) with stirring. Undissolved material was filtered off at the pump, and the filtrate was evaporated to dryness under reduced pressure.

A solution of DCCD (1.65 g, 8 mmole) in t-butanol (30 ml) was added dropwise, over a period of 3-4 hours, to a warm stirred solution of z^8AMP (triethylammonium salt, 1.2g, 2mmole) in a mixture of water (20 ml) and t-butanol (20 ml) containing redistilled morpholine (0.68 ml, 8 mmole). The solution was maintained at approximately 50° overnight, and the products monitored by tlc in solvents A and D. If the z^8AMP appeared not to have been largely converted to a product with elevated mobility on these plates, a further quantity of DCCD (0.825 g, 4 mmole) in t-butanol (15 ml) was added, and stirring continued for a further 24 hours. The reaction mixture was evaporated under reduced pressure ($T < 45^{\circ}$ C) until most of the t-butanol had been removed and white crystals of the N, N'-dicyclohexyl urea were evident. These were filtered off after addition of water (30 ml), the filtrate was extracted with diethyl ether (3 x 30 ml), followed by a further filtration, if necessary, of the aqueous phase. Evaporation of water

under reduced pressure produced a yellow residue of the phosphomorpholidate.

Separately, 88% orthophosphoric acid (0.382 ml, 6 mmole) and tri-n-butylamine (1.43 ml, 6 mmole) were dissolved in pyridine (20 ml) and the solution evaporated in vacuo. The procedure was repeated twice. The crude phosphomorpholidate was dissolved in pyridine (20 ml) and a similar series of evaporations was performed. Both products were again separately dissolved in pyridine (20 ml) and the tributylammonium phosphate solution was added slowly to the phosphomorpholidate with stirring. The mixture was left in the dark for 60 hours, after which tlc in solvent systems A and D showed conversion to a product with very low mobility compared to z^8AMP, but which possessed the same UV spectrum. Pyridine was removed under reduced pressure ($T < 45^oC$), the residue dissolved in water (200 ml) and the solution applied to a 50 x 2.5 cm DEAE Sephadex A25 column (HCO_3^- form). Washing and elution were as described for br^8AMP except that the composition of the linear gradient of triethylammonium bicarbonate was altered (0.05 - 0.20 M, pH 8.7, 2 l). The reaction sequence used for the preparation of z^8ADP is shown in Figure 2.3 and a typical elution profile of the nucleotide in Figure 2.4. The yield of diphosphate from monophosphate was in the range of 40% over a number of preparations whilst the spectral characteristics were in all ways identical to that of the monophosphate.

The sodium salt of z^8ADP was obtained qualitatively by elution through SE Sephadex C25 as described for z^8AMP.

2.2.6 Synthesis of 8-azido-adenosine

The triethylammonium salt of z^8AMP (15 mg) was incubated for 18 hours at 37^oC in tris-HCl buffer, pH 9.0 (50 mM, 1 ml) containing $MgCl_2$ (0.001 M) and alkaline phosphatase (350 U/ml, 10 µl). Silica tlc in solvent C showed conversion to a new major product with a high mobility. The incubation mixture was layered on to a Sephadex G50 column (30 x 1 cm) which was eluted with water. Those fractions containing UV-absorbing material were pooled, evaporated to dryness

Figure 2.3

The synthesis of 8-azido-adenosine diphosphate from adenosine monophosphate

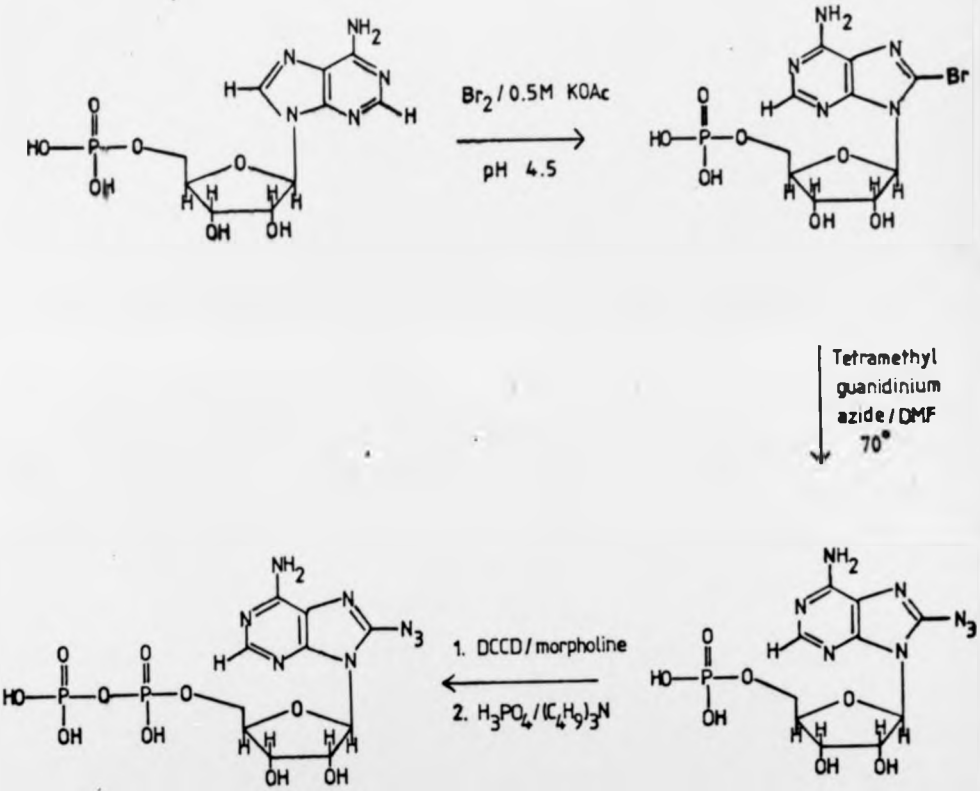
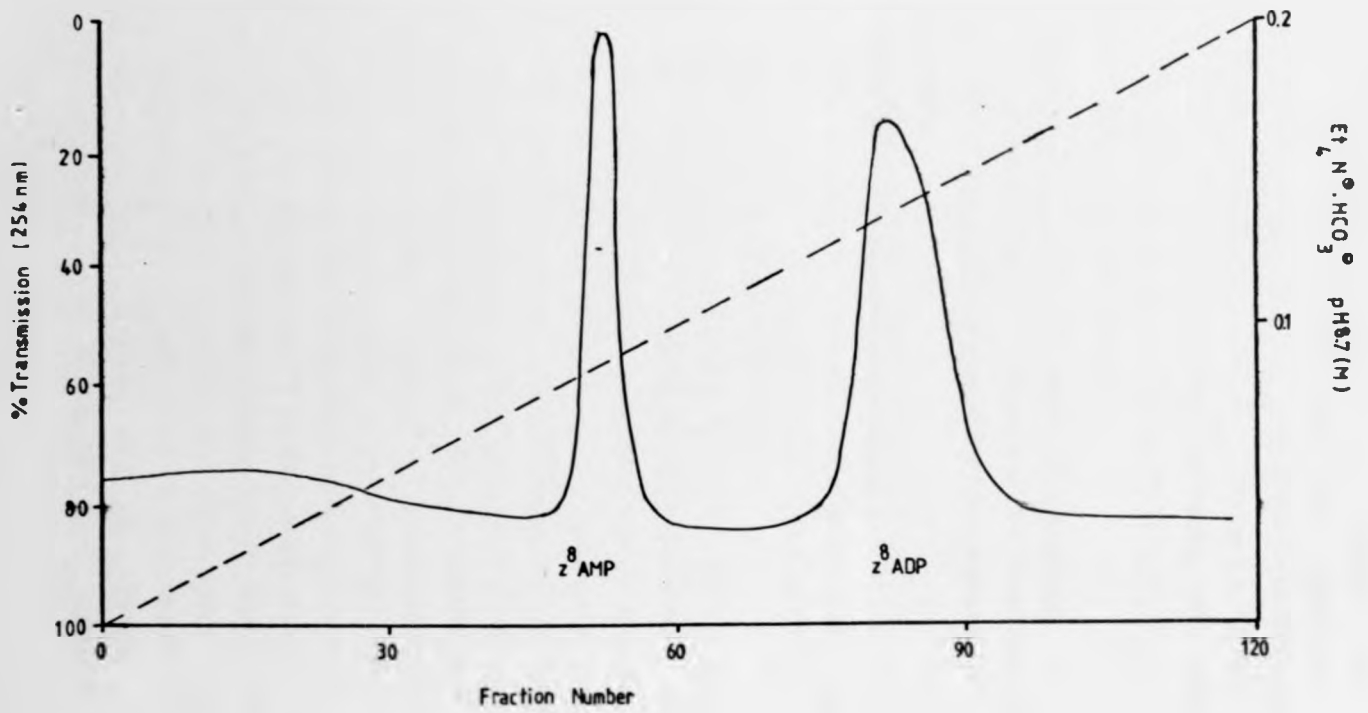

Br2 / 0.5M KOAc
pH 4.5
Tetramethyl guanidinium azide / DMF 70°
1. DCCD / morpholine
2. H3PO4 / (C4H9)3N

Figure 2.4

Typical elution profile from DEAE Sephadex A25 in the purification of 8-azido-adenosine diphosphate, using a linear gradient of triethylammonium bicarbonate, pH 8.7


% Transmission (254 nm)
0
20
40
60
80
100
0
30
60
90
120
Fraction Number
z^8AMP
z^8ADP
0.2
0.1
Et$_4$N$^\oplus$.HCO$_3^\ominus$ pH8.7 (M)

and recrystallised from hot water to yield 7.4 mg (80%) of z^8Ado; m.p. 196° C (lit. 226-229 dec. (Holmes and Robins, 1965)); pH 1, λ_{max} 281.5 nm (ϵ, 17,600), pH 7, λ_{max} 281.5 nm (ϵ, 13,500); pH 11, λ_{max} 281.5 nm (ϵ, 13,500): lit., pH 1, λ_{max} 281 nm (ϵ, 17,300; pH 11, λ_{max} 281 nm (ϵ, 13,500) (Holmes and Robins, 1965) (see Figure 2.5). Found: C, 39.61; H, 4.15; N, 36.4; $C_{10}H_{12}N_8O_4$ requires C, 38.96; H, 3.92; N, 36.35%.

2.2.7 Synthesis of 8-azido-2-[^{3}H]-adenosine diphosphate

This was prepared in 28.4% overall yield by the procedures outlined above for z^8ADP. The specific activity of the final product was 10 mCi/mmole.

2.2.8 Synthesis of 8-azido-inosine diphosphate

The method presented for deamination of adenosine was adapted from that of Eaton (1973), based on the original report by Holmes and Robins (1964) for conversion of br^8A to br^8I.

z^8ADP (trisodium salt, 100 mg) was dissolved in 10% v/v acetic acid (3 ml) and cooled to 0° C. Over a period of about 2 hours, solid sodium nitrite (0.3g) was added to the stirred solution. Analysis of the reaction product showed a shift in the absorbance maximum from 281.5 to 275 nm, whilst silica tlc in solvent D showed the production of a new spot with a mobility approximately 0.5 that of z^8ADP. The solution was lyophilised and pumping continued until all traces of acetic acid had been removed. The residue was dissolved in water (60 ml) and purified in the normal way (as described for z^8ADP) on a column (20 x 0.75 cm) of DEAE Sephadex A25 (HCO_3^- form) (see Figure 2.6). Fractions containing z^8IDP were pooled, lyophilised and converted to the sodium salt as described for z^8AMP. Yield (in terms of O.D. units) = 70%; pH 1, λ_{max} 274 nm (ϵ, 14,225); pH 7, λ_{max} 275 nm (ϵ 14,225); pH 11, λ_{max} 281 nm (ϵ, 11,640): lit. for z^8cIMP, pH 1, λ_{max} 273 nm (ϵ, 15,700); pH 11, λ_{max} 280 nm (ϵ, 13,000) (Miller et al., 1973) (see Figure 2.7); ν_{max}(Nujol) 2160 cm^{-1} (strong).

Figure 2.5

Ultra-violet spectra of z^8Ado at pH 1 (A) and at pH 11 (B)

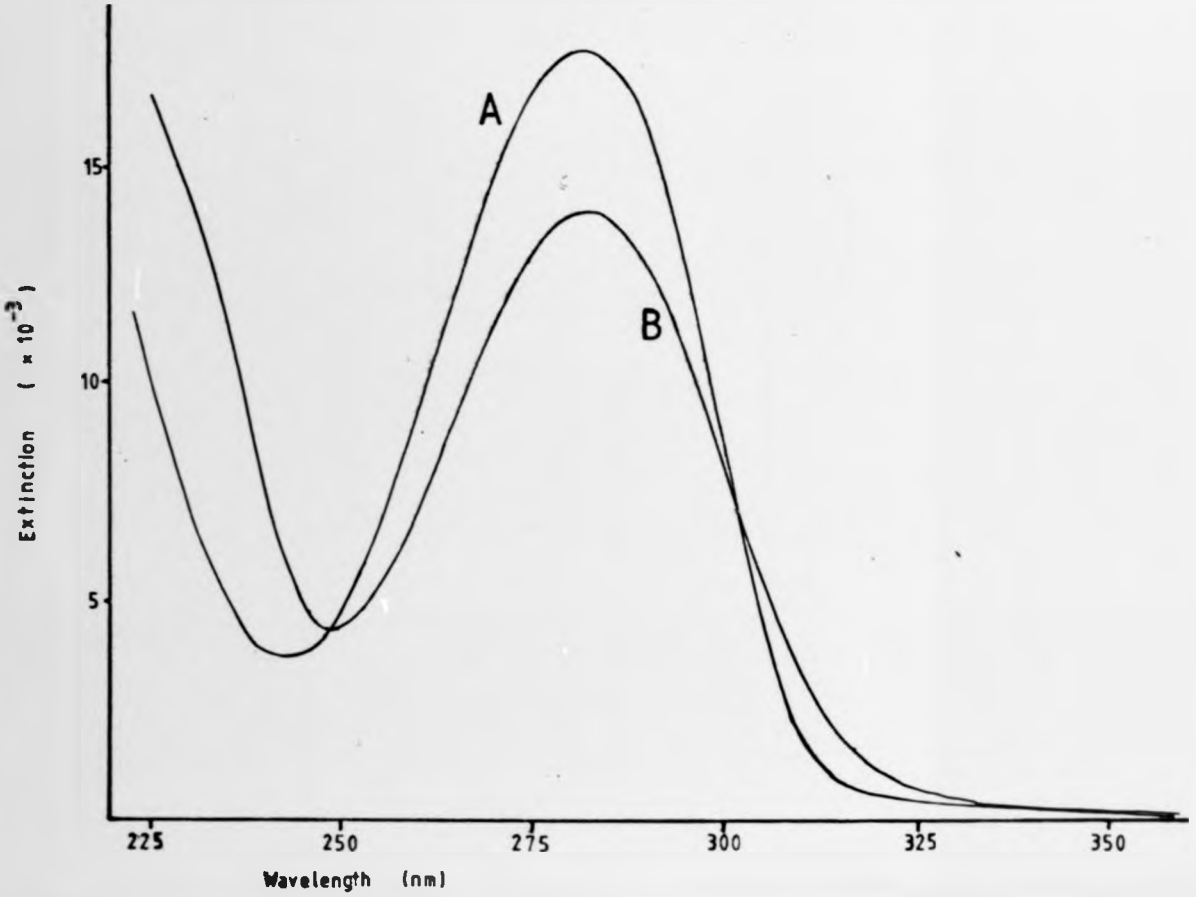

A
B
15
10
5
Extinction (× 10⁻³)
225
250
275
300
325
350
Wavelength (nm)

Figure 2.6

Typical elution profile from DEAE Sephadex A25 in the purification of 8-azido-inosine diphosphate, using a linear gradient of triethylammonium bicarbonate, pH 8.7

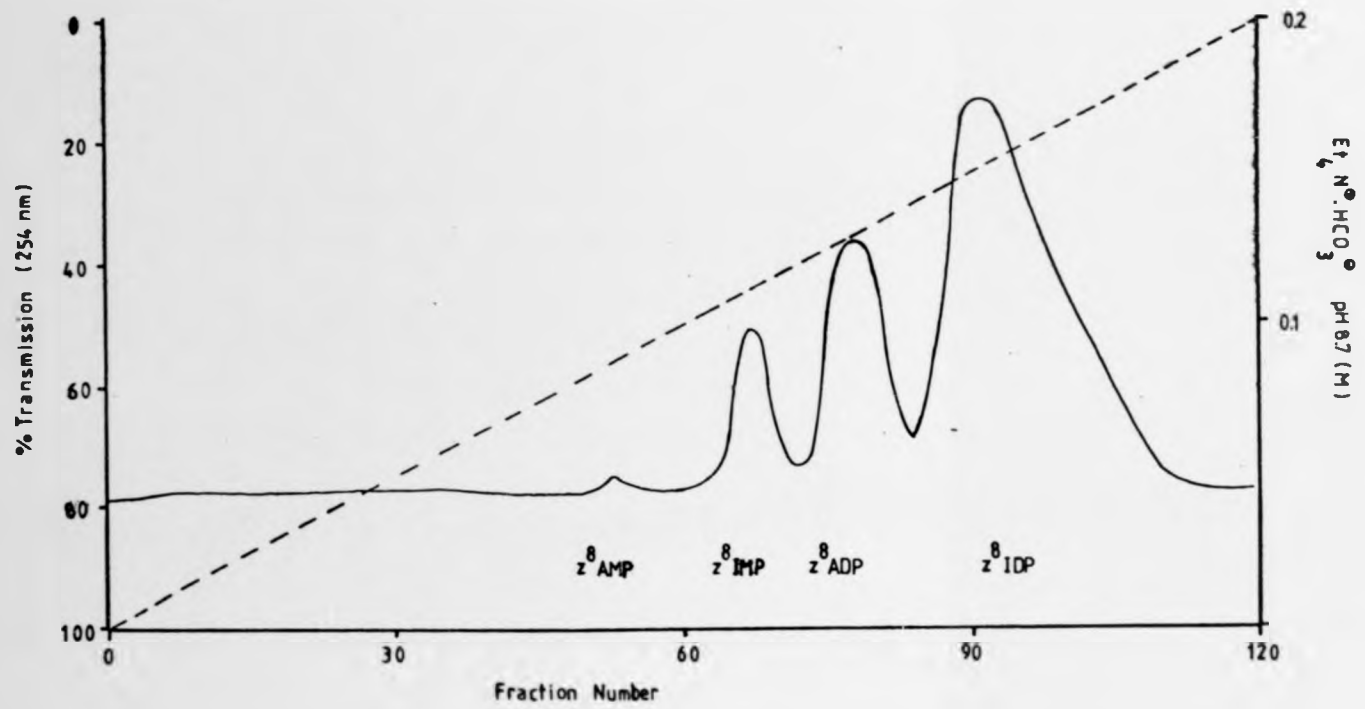
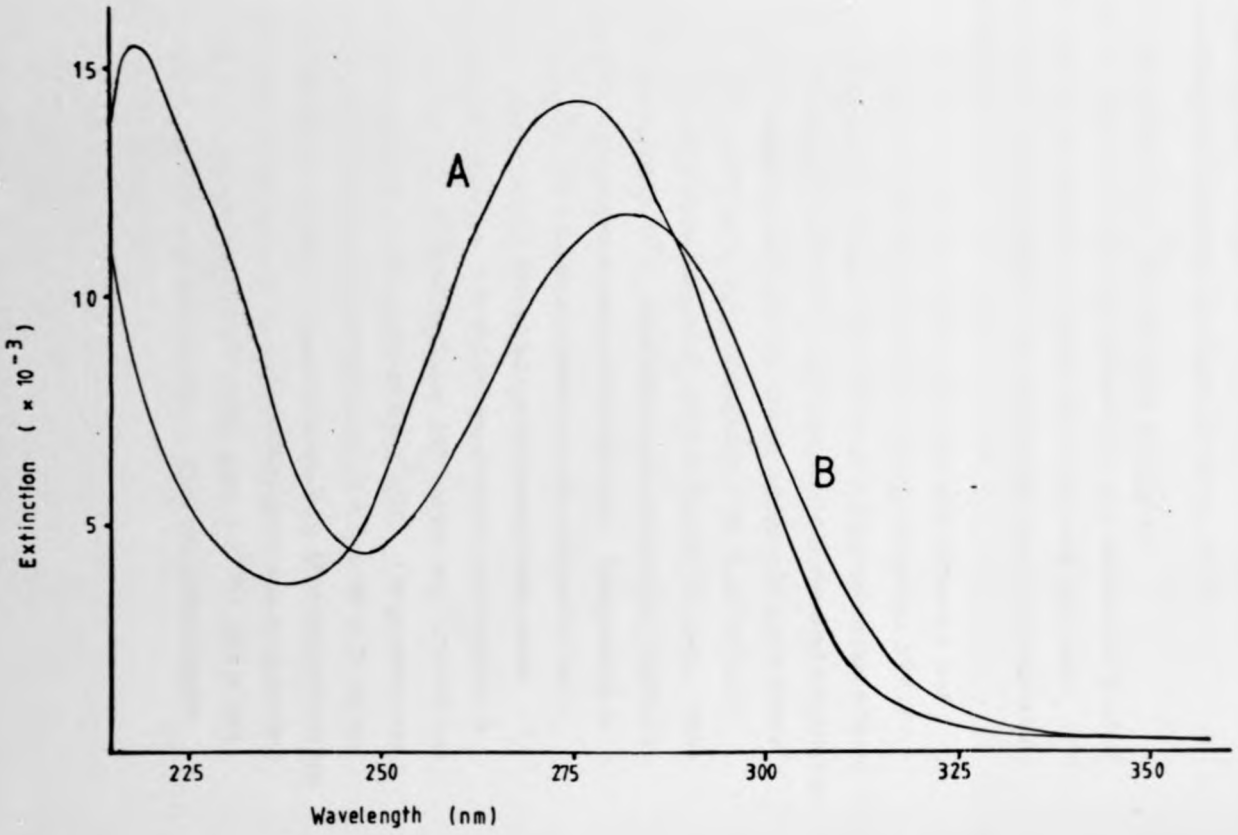

% Transmission (254 nm)
0
20
40
60
80
100
0
30
60
90
120
Fraction Number
0.2
0.1
Et4N+.HCO3- pH8.7 (M)
z8AMP
z8IMP
z8ADP
z8IDP

Figure 2.7

Ultra-violet spectra of z^{9}IDP at pH 1 (A) and at pH 11 (B)


15
10
5
Extinction (× 10⁻³)
A
B
225
250
275
300
325
350
Wavelength (nm)

2.2.9 Synthesis of 8-azido-inosine

This was performed essentially as described above for z^8Ado, except that the product was purified by paper chromatography in solvent G after the Sephadex G-50 step. Yield (in terms of O.D. units) 78.8%; m.p. dec > 200°; mass spectrum m/e $(B + H - N_2)^+$ 149. Mass spectra of azides are rarely seen to display a molecular ion; commonly loss of nitrogen is observed (Gurst, 1971).

2.2.10 Synthesis of α-[^{32}P] adenosine diphosphate

α-[^{32}P]-ADP was found to be unobtainable as a commercial product, so the following procedure was devised (Cartwright and Hutchinson, 1977) and was found to be applicable to the preparation of all ribonucleoside diphosphates.

α-[^{32}P]-ATP (0.25 mCi, specific activity 10.3 Ci/mole) was transferred to a small reaction vessel containing unlabelled ATP (1 μmole) by washing with water. The ATP was lyophilised so that ethanol (in which the labelled nucleotide is supplied) could be removed completely. The following reagents were added in order: sodium phosphate buffer, pH 7.5 (0.05 M, 0.728 ml), $MgCl_2$ solution (0.1 M, 0.067 ml) and glucose (0.5 M) in sodium phosphate, pH 7.5 (0.05 M, 0.2 ml). After 5 minutes incubation at 25° C, yeast hexokinase (1600 U/ml, 0.005 ml) was added and the incubation continued for an hour. Cellulose tlc in solvent D showed that complete conversion to the diphosphate had occurred. Ethanol (1 ml) was added and the precipitated protein removed by centrifugation. The diluted supernatant was applied to a column (25 x 1.1 cm) of DEAE Sephadex A25 and the α-[^{32}P]-ADP was then eluted and worked-up as described for z^8ADP. The gradient was formed from triethylammonium bicarbonate, pH 8.9, (0.05-0.25 M) and a peak of radioactivity eluted at approximately 0.16 M triethylammonium bicarbonate. Exchange of cation to the sodium form was as described for z^8AMP, and lyophilisation of the eluate gave α-[^{32}P]-ADP in 88% yield (specific activity 0.23 mCi/μmole). This was stored frozen in water at -20° C until used.

It was found that complete conversion of the three triphosphates, CTP, UTP and GTP, to the diphosphates could be achieved by addition of twice the quantity of enzyme and incubation for 3 hours at 50° C (monitored by silica tlc in solvent E). Lower temperature (37° C) produced only complete conversion of UTP to UDP, whilst even four times the amount of enzyme proved insufficient to produce any of the diphosphates at 25° C.

Conditions used for identification of the integrity of the ^{32}P label at the α-position of the nucleotide are given in the legend to Table 2.1, where the results of this experiment are also presented.

2.2.11 Identification of products of reaction between z^8AMP and a dithiol

Dithiothreitol (33 mg, 0.2 mmole) was added in portions to a solution of bis(triethylammonium) z^8AMP (25 mg, 0.04 mmole) in aqueous triethylammonium bicarbonate, pH 8.7, (0.01 M, 5 ml). Rapid evolution of gas occurred whilst the mixture was stored in the dark. After 18 hours, evaporation of solvent in vacuo was followed by purification of the product on a column (20 x 0.75 cm) of DEAE Sephadex A25 as described for z^8AMP, using a linear gradient of triethylammonium bicarbonate, pH 8.7, (0-0.15 M). The lyophilised product was identical in both its UV absorbance characteristics and its mobility on tlc in solvent D to the n^8AMP prepared by hydrogenation of z^8AMP using Pd/C as described by Holmes and Robins (1965) for z^8Ado. The n^8AMP was not purified further, but was dephosphorylated as described for z^8AMP above to give colourless crystals of n^8Ado; yield 19 mg (80%); m.p. 188-192° C (lit. 180-185° (Holmes and Robins, 1965)); pH 1, λ_{max}, 269 nm (ε 13,200); pH 11, λ_{max}, 274 nm (ε, 16,400) (see Figure 2.8): lit. pH 1, λ_{max}, 270 nm (ε, 13,500); pH 11, λ_{max} 273 nm (ε, 16,400) (Holmes and Robins, 1965): mass spectrum m/e M^+ 282, $(B+H)^+$ 150. Found: C, 42.48; H, 5.36; N, 29.7; $C_{10}H_{14}N_6O_4$ requires C, 42.55; H, 5.00; N, 29.8%.

Table 2.1

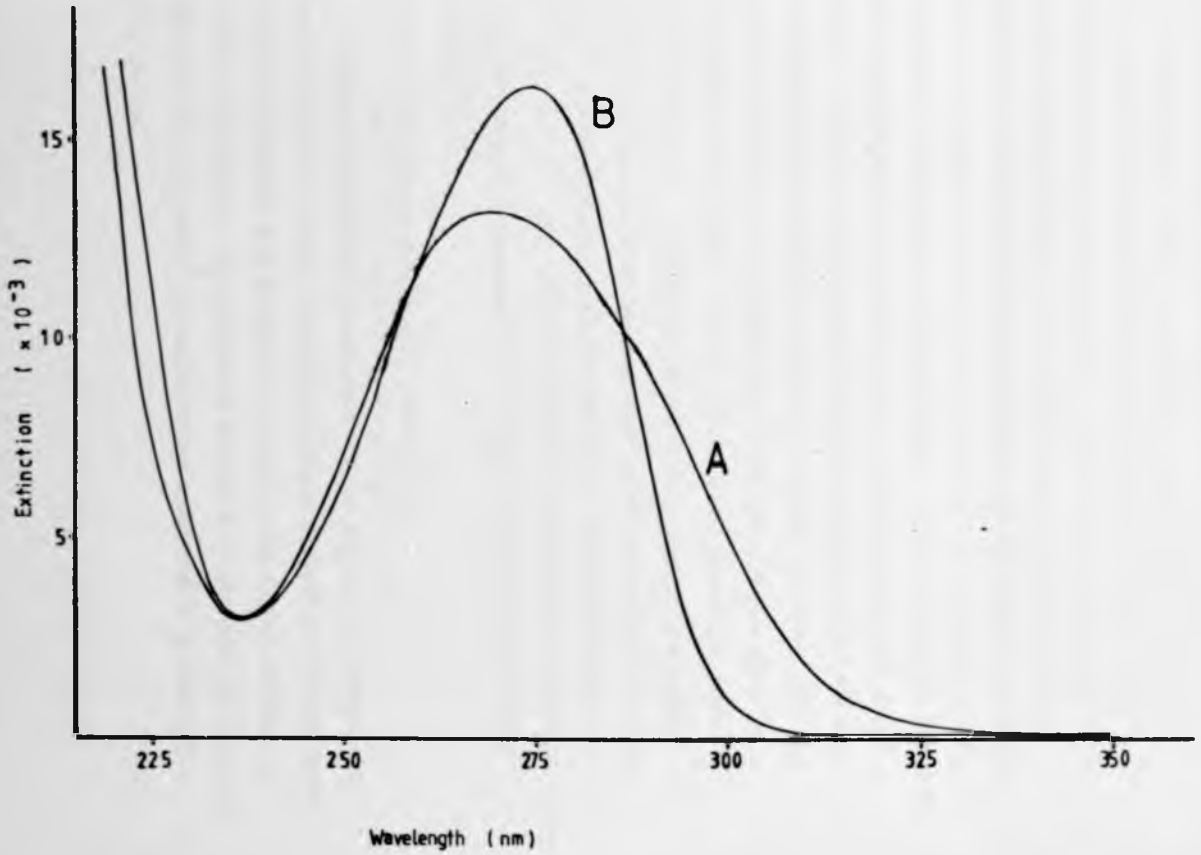
Positional integrity of ^{32}P label in synthetic α-[^{32}P)-ADP by PNPase-catalysed polymerisation

% phosphate released from ADP	% of input counts incorporated with A_n
30.6	31.4

Polymerisation mixtures contained the following: ADP (5.3 mM), $MgCl_2$ (3 mM), tris-HCl, pH 9.0 (50 mM), polynucleotide phosphorylase (0.3 U) and ^{32}P-labelled ADP (5.5×10^4 cpm), and were incubated at 45° for 30 minutes. Inorganic phosphate release was assayed by precipitation of aliquots of incubation mixture on Whatman GF/A filter discs by passage through 5% trichloroacetic acid (2 x 15 minutes), then briefly rinsed in ethanol followed by ether. Radioactivity on the discs was determined in toluene based scintillant. The close correspondence between the two values demonstrates the required integrity at the α-position.

Figure 2.8

Ultra-violet spectra of n^8Ado at pH 1 (A) and at pH 11 (B)


B
A
15
10
5
Extinction (x 10⁻³)
225
250
275
300
325
350
Wavelength (nm)

2.2.12 Preparative photolysis of z^8AMP

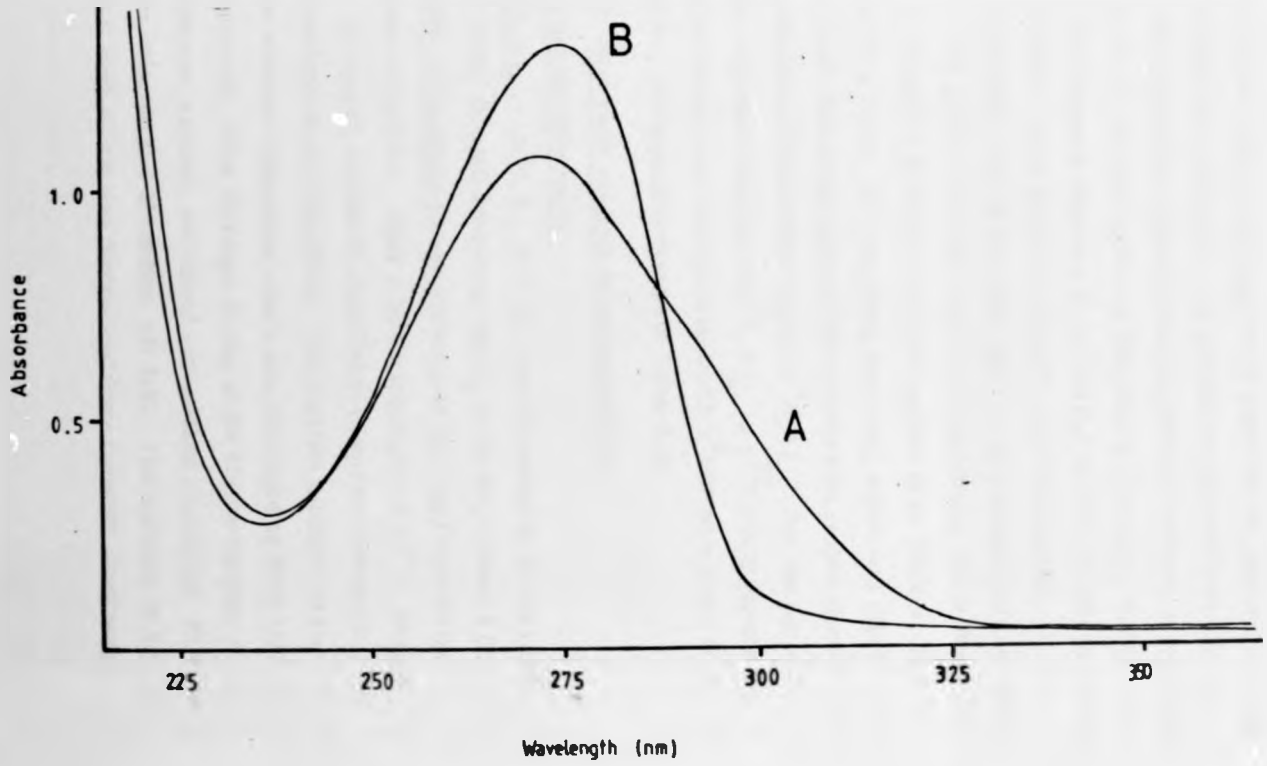
A solution of z^8AMP in deoxygenated water (triethylammonium salt, 0.1 M, 1 ml) was photolysed in a quartz cuvette behind a pyrex glass filter (opaque to wavelengths < 280 nm) for 15 hours at 2° C until the UV spectrum did not change appreciably. The wavelength maximum changed from 281 nm to ca. 270 nm while there was a noted increase in absorbance above 320 nm (the solution turned deep red). Silica tlc in solvents A, D and F showed that a large amount of material remained at the origin, whilst one or two spots were seen to move more slowly than starting material. The material was applied to a column of DEAE Sephadex A25 and eluted with a series of linear gradients of triethylammonium bicarbonate, pH 8.7, up to a maximum of 0.67 M, and the products (as monitored by UV absorbance) pooled and lyophilised as described above for z^8AMP. Most of the highly coloured material remained on the column even at concentrations of 0.5 M elutant. The first major product from the column purification was investigated in some detail. Silica tlc in solvent A showed that this product was identical in mobility to n^8AMP prepared as above by hydrogenation of z^8AMP. The UV spectra (see Figure 2.9) were virtually identical. Dephosphorylation of this product by alkaline phosphatase etc. (as described above in Section 2.2.6) gave a product identical in tlc mobility to n^8Ado in sdvent A. Mass spectrum m/e M^+ 282, $(B+H)^+$ 150. Other products from the column purification were studied briefly, but appeared to be complex high molecular weight compounds.

2.2.13 Preparation of $(z^8A,A)_n$ and $(z^8I,I)_n$

A typical preparation of a sample of $(z^8A,A)_n$ and its purification are given below. Details of particular polymers in terms of their input ratio of nucleotides will be found in legends to the tables and figures in which they appear, but the general details of the process are identical for both $(z^8A,A)_n$ and (z^8I,I) and for their synthesis from radioactive precursors.

Figure 2.9

Ultra-violet spectra of major product from solution photolysis of z^8AMP, at pH 1 (A) and at pH 11 (B)


B
A
1.0
0.5
Absorbance
225
250
275
300
325
350
Wavelength (nm)

The polymerisation mixture (10ml) consisted of tris-HCl, pH 9.0, (0.05 M), ADP (0.01 M), z^8ADP (0.05 M), manganese chloride solution (0.004 M) and E. coli polynucleotide phosphorylase (30 U). After incubation in the dark at 45° C for 24 hours, the solution was extracted by shaking with an equal volume of iso-amyl alcohol/chloroform (3:1 v/v) followed by low-speed centrifugation to separate the phases. The top aqueous layer was withdrawn, the procedure repeated three more times, and the aqueous layer finally dialysed for 24 hours in Spectrapor 2 tubing (M.W. cut-off 12,000-14,000; Raven Scientific, Suffolk, U.K.) against the following solutions (2 l): (i) 0.5 M NaCl, 0.001 M tris-HCl, 0.001 M EDTA, pH 7.4; (ii) 0.5 M NaCl, 0.01 M tris-HCl, pH 7.4; (iii) 0.1 M NaCl, 0.01 M tris-HCl, pH 7.4; (iv) double distilled water (twice). The product (40-50% yield) was lyophilised, the fluffy white polymer dissolved in water (1 ml) and applied to an Ultrogel AcA 34 column (80 x 2 cm). UV-absorbing material, which was obtained at the void volume following elution of the column with double distilled water, was collected and lyophilised to give $(z^8A,A)_n$. For the purposes of a nearest neighbour analysis of $(z^8A,A)_n$, α-[^{32}P]-ADP (synthesis described above) was copolymerised with z^8ADP on a small scale. For conditions, see legend to results in Table 2.5.

2.2.14 Base ratio analysis of polynucleotides

(a) By nuclease digestion

(i) $(z^8A,A)_n$.- $(z^8A,A)_n$ (0.1 mg) was dissolved in bicine buffer, pH 8.0 (0.02 M, 0.1 ml) containing $MgCl_2$ (0.01 M), RNase A (0.1 mg), Crotalus adamanteus phosphodiesterase (0.1 mg) and alkaline phosphatase (3.5 U). After 6 hours incubation at 37° C, analysis by tlc (silica gel, solvent C) showed that complete hydrolysis to the nucleosides had occurred. The reaction mixture was applied as a band to Whatman 3MM paper which was developed by downward elution with solvent B. After thorough drying of the chromatogram, the spots were located, excised and eluted with sodium cacodylate (0.01 M) in sodium chloride (0.1 M) buffer, pH 7.0. The amounts of Ado and z^8Ado in each eluate was determined from their UV absorbance.

(ii) $(z^{8}I,I)_n$.- $(z^{8}I,I)_n$ (0.1 mg) was dissolved in tris-HCl, pH 7.5 (0.05 M, 0.1 ml) containing RNase T_1 (1.86×10^5 U/ml, 0.015 ml) and alkaline phosphatase (3.5 U) and incubated at 37° C for 36 hours. Analysis of the product by tlc (silica, using solvent G) showed complete hydrolysis to the nucleosides and very good separation of the two products. Paper chromatographic separation of the nucleosides and quantitation was as described above for $(z^{8}A,A)_n$ except that the chromatogram was developed in solvent G.

(b) By alkaline hydrolysis

Azido polymer (1-2 O.D.$_{\lambda_{max}}$ units) was dissolved in 0.3 M sodium hydroxide (1 ml) and incubated overnight at 37° C until all hyperchromism of the spectrum had ceased. The spectrum of liberated nucleotides was analysed from the knowledge of their respective extinction coefficients at a number of wavelengths, giving the same ratios of the polymer directly. The method used allows the construction, from a theoretical standpoint, of the spectrum of hydrolysed co-polymer of any pre-determined composition, and can thus be tested for validity against a polymer, the base ratio of which is known by an independent method. Such an analysis may also be made on an enzymically hydrolysed polymer if the protein has been removed or does not interfere with the spectrum.

2.2.15 End group determination of $(z^{8}A,A)_n$

The procedures of Randerath et al. (1972) and Cory et al. (1976) were adapted. Sodium [^{3}H]-borohydride (661 mCi/mmole, 25 mCi) was dissolved in sodium hydroxide solution (0.1 M, 0.378 ml) to give a 0.1 M solution. This was stored in 50 µl portions at -20° and was stable for one month.

(a) Preparation of [^{3}H]-labelled nucleoside trialcohols

To a solution of nucleosides (6 mmole) in water (0.03 ml) was added aqueous sodium periodate (0.0025 M, 0.005 ml) and the mixture incubated at room temperature for 90 minutes in the dark. This was followed by addition of $NaB[^3H]_4$ solution (0.1 M, 0.001 ml) with a further period of 90 minutes incubation in the dark. To destroy excess borohydride, acetic acid (0.1 M, 0.025 ml) was added (CARE - tritium gas evolved), and after 30 minutes in the fume cupboard, the solution was dried in air, redissolved in water (0.03 ml) and stored frozen at

-20° C until required. Silica tlc of the products in solvents B and C showed complete conversion of the ribonucleosides to products of significantly lower mobility.

(b) 3'-end [^{3}H]-labelling of $(z^8A,A)_n$

A sample of $(z^8A,A)_n$ (50 µg) was dissolved in EDTA solution, pH 8.0 (0.001 M, 0.1 ml) and oxidation of the 3'-terminal sugar was effected by addition of sodium periodate solution (0.025 M, 0.03 ml) with subsequent incubation in the dark for 60 minutes at room temperature. After addition of sodium bicine buffer (0.6 M, 0.02 ml) and chilling to 0° C, sodium [^{3}H]-borohydride (0.006 M in 0.006 M NaOH, 0.09 ml) was introduced, with further room temperature incubation in the dark. Excess borohydride was destroyed after 90 minutes by acetic acid (1 M, 0.025 ml) and after all evolution of gas, was lyophilised. The sample was dissolved in ammonium carbonate, pH 7.5 (0.1 M, 1 ml), chromatographed on a column of Sephadex G75 (90 x 2 cm) and the fractions containing radioactivity at the void volume were pooled and lyophilised to remove ammonium carbonate. The residue was hydrolysed to nucleosides by dissolution in the following mixture (0.2 ml total) and incubation at 37° C overnight: sodium bicine (0.6 M, 0.01 ml), RNase A (10 mg/ml, 0.02 ml), Crotalus adamanteus phosphodiesterase (10 mg/ml, 0.02 ml), alkaline phosphatase (350 U/ml, 0.01 ml), $MgCl_2$ solution (0.1 M, 0.02 ml) and water (0.12 ml). Silica tlc in solvent G showed complete hydrolysis to nucleosides using Ado and z^8Ado as standards. The reaction mixture was applied as a band to a sheet of silica gel (20 x 20 cm), developed in solvent G and fluorographed at -70° C according to the method of Randerath (1970) using Kodak X-Omat H X-ray film. The nucleoside trialcohols as prepared above were run as markers.

2.2.16 Complex formation of $(z^8A,A)_n$ and $(z^8I,I)_n$ with complementary polynucleotides

The method of continuous variation (Job, 1928) as applied to the determination of polynucleotide complex formation (Felsenfeld et al., 1957) was used to investigate the possible interaction between the synthetic copolyribonucleotides $(z^8A\ A)_n$ and $(z^8I\ I)_n$ with their complementary counterparts U_n and C_n respectively.

A series of mixtures at fixed total nucleotide concentration were prepared under given salt conditions. These were heated to 70^{o} for 10 minutes and then allowed to cool slowly. The solutions were stored at 4^{o} C for a minimum of 2 weeks to ensure total equilibration, before observation of their spectral characteristics.

Mixtures of polyribonucleotides were subjected to thermal denaturation analysis by monitoring of their hyperchromicity at a fixed wavelength as a function of temperature. Series of mixture (at 1:1 and 2:1 input ratios of polymer) were prepared at differing ionic strength for this investigation.

Details of the individual conditions for a given experiment are given in the legends to the relevant figures and tables.

2.3 Results and Discussion

2.3.1 8-Azido-nucleotides

The synthetic methods described were found to be easy to apply and consistent in terms of reproducibility in both yield and purity. Lee and Kaplan (1975) have described a simple procedure for preparation of br^{8}AMP in high yield involving chloroform extraction of unreacted bromine and lyophilisation of the aqueous phase. However, it was not useful in the present study, since only column purified products were found to be suitable for the introduction of the azido group under anhydrous conditions. Previous studies on the introduction of the azido group into the 8-position of the adenine base utilised sodium azide in DMF as the nucleophile (Holmes and Robins, 1965: Muneyama et al., 1971). In this study, such conditions gave no appreciable substitution because, in our hands, sodium azide appeared totally insoluble in the solvent. A commercial product, tetramethyl guanidinium azide (Papa, 1966) was highly soluble in organic solvents and gave consistent substitution of bromine in good yield. In publications concerning the same reaction that have appeared since the initiation of this work, a variety of DMF-soluble azides have been described, e.g. triethylammonium azide (Haley, 1975; Boos et al., 1978), tributyl-ammonium azide (Koberstien et al., 1976; Wagenvoord et al., 1977) and hydrazoic acid in the presence of triethylamine (Schäfer et al., 1976). These involve time-consuming synthesis and, in one case, a potentially hazardous reagent is used. Since the reported yields are no better than those given here, it is suggested that the use of tetra-methyl guanidinium azide is of most convenience for the synthesis of azido nucleotides.

Both z^{8}Ado and z^{8}Ino nucleotides show strong infra-red absorption around 2150 cm^{-1}, a region characteristic of asymmetric stretching for covalent azides (Lieber et al., 1966). This fact, together with their facile photolysis (see below) argue against the existence of the tetra-azole form (by ring closure with N-7) as found by Wiegand and Kaleja (1976) for 2-azido-inosine nucleosides.

The introduction of the azido group into the 8-position of purine nucleosides and nucleotides has been assumed to force the base to take up the syn conformation (Haley and Hoffmann, 1974), but no sound basis for this assumption has been laid. The present work provides both indirect and direct evidence that this is indeed the case. It is instructive to consider the similarities between the azido group and the bromine atom, since it has been shown conclusively that br^8A nucleosides and nucleotides exist in the syn conformation (Tavale and Sobell, 1970; Jordan and Niv, 1977). Thus the effective radius of azide and bromide are comparable from crystal studies, and their electronic properties e.g. electronegativity and dipole moment, are similar (Treinin, 1971). The azido group is linear, and studies in covalent azides have shown that the bond distance between C and the terminal N is approximately 3.8Å. The $C\text{-}\overset{\alpha}{N}\text{-}\overset{\beta}{N}$ - bond angle is usually 120°, but dipole moment studies in p-chloro and p-bromophenyl azides show that the dipoles nearly cancel each other, and the bond angle is thus assumed to be much wider. This is probably because the α-nitrogen possesses lone-pairs of electrons in p-orbitals and can almost certainly interact by electron-delocalisation with the aromatic ring, thus causing a bond angle closer to 180°. In the aromatic azido-nucleotide, this means that the bulky azido group is probably not able to bend away from severe non-bonded contact with substituents of the furanose ring, with the result that relief of steric crowding can be achieved most effectively by adoption of the syn conformation. Model-building studies also show this as likely to be the case.

The C.D. spectra of z^8Ado and z^8Ino determined in this investigation are shown in Figure 2.10, compared with Ado and Ino. They both show a small positive Cotton effect with a broad band centred around 270 nm, similar to br^8Ado and distinctly different from the unsubstituted nucleosides. Rogers and Ulbricht (1970) and Ikehara et al. (1972) have made extensive studies of 8-substituted adenine nucleosides and nucleotides and have concluded that C.D. is a very sensitive spectro-

Figure 2.10

Circular dichroism spectra of z^8Ado and z^8Ino

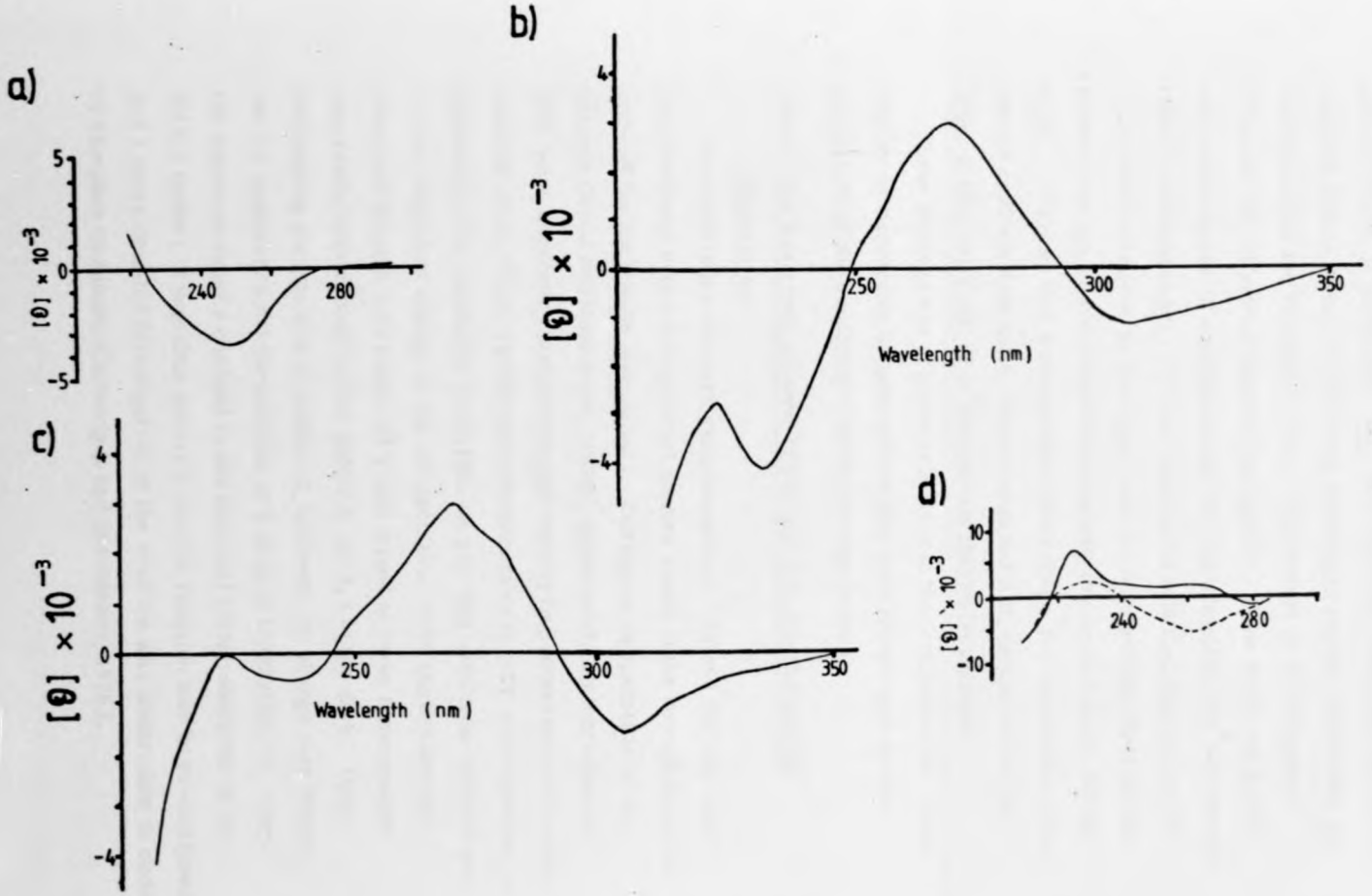
Spectra were recorded in 0.01 M sodium cacodylate, 0.1 M sodium choride at 20°, and are compared with published spectra

(a) Ino (Delabar and Guschlbauer, 1973)

(b) z^8 Ino

(c) z^8Ado

(d) Ado (-----) and br^8Ado (———) (Ikehara et al., 1972)


a)
b)
c)
d)
[θ] × 10⁻³
Wavelength (nm)
240
250
280
300
350
5
4
0
-4
-5
10
-10

scopic method for detecting conformational differences about the glycosyl bond. In particular, anti conformations are characterised by negative Cotton effects in this long wavelength region, whilst for syn conformations the reverse is true. Substitution by 5'-phosphate enhances the difference between the spectra. Thus it can be stated with some degree of confidence that the conformation of z^8Ado nucleotides in solution is syn. It is of interest to note that the results of C.D. determinations on Guo (anti) and its 8-substituted derivatives (known to be syn) have yielded substantially the same results (Miles et al., 1971), so that it would seem likely that z^8Ino nucleotides adopt the syn conformation also. When compared with unsubstituted Ino (Fig. 2.10), the C.D. of z^8Ino reveals this to be the case.

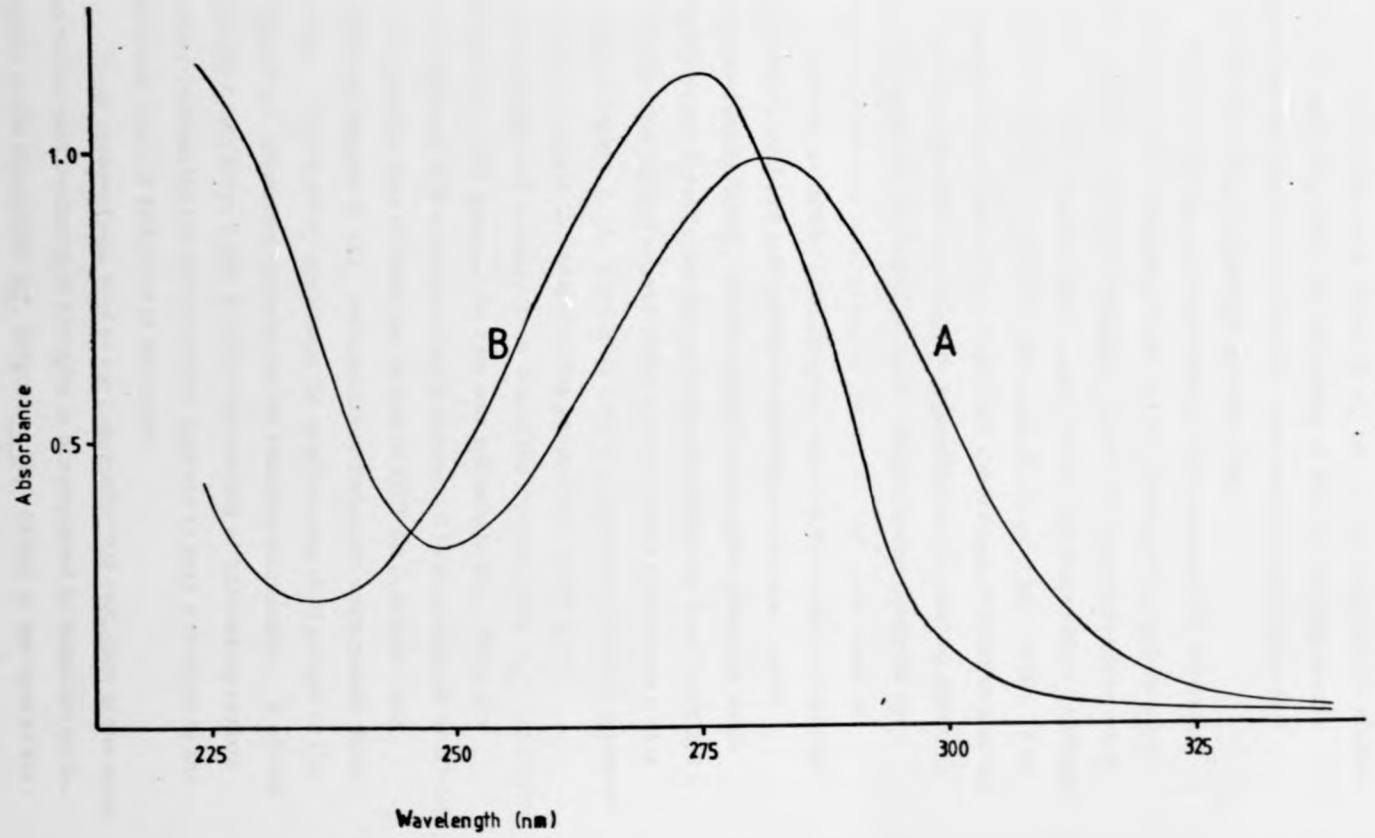
These findings are of great relevance to the interpretation of the results of interaction of these nucleotides with PNPase and on the properties of the copolymers produced (see below).

2.3.2 The Reactions between Thiols and 8-Azido-Nucleoside Derivatives

The occurrence of a dark reaction when z^8ADP and PNPase were incubated was noted during initial studies aimed at the homopolymerisation of the nucleotide (see below). During the preparation of the enzyme (Wood and Hutchinson, 1976), substantial concentrations of DTT were necessary to maintain high activity by preservation of a fully reduced state. Thus, significant concentrations of DTT were present in polymerisation incubation mixtures. A gas was seen to be evolved and a time-dependent change in the UV spectrum of the nucleotide was observed (Figure 2.11) when DTT was mixed at room temperature separately with z^8AMP in tris buffer at pH 8.2 in the dark. Upon performing the reaction in methanol, however, no change was seen in the UV spectrum until the addition of a drop of triethylamine, when the spectrum rapidly changed to one identical to that observed in the pH 8.2 buffer. It was thus apparent that the reaction was base-catalysed and a more detailed investigation of the reaction was undertaken in order to elucidate its nature (Cartwright and Hutchinson, 1976).

Figure 2.11

Change in ultra-violet spectrum of z^8AMP after reaction with dithiothreitol. Spectra were determined 0.05 tris-HCl, pH 8.2 before (A) and after (B) addition of dithiothreitol to a final concentration of 0.26 mM


1.0
0.5
Absorbance
B
A
225
250
275
300
325
Wavelength (nm)

As can be seen from Figure 2.11, the UV spectrum of the product of DTT action has very low absorption at 300 nm, so that kinetic studies were conveniently made by observation of the change in absorbance at this wavelength against time.

Previous studies had shown that in high temperature reactions, azides could be reduced by thiols to the corresponding amines via a free-radical mechanism (Shingaki, 1963) and that the reaction could occur when catalysed by Cu(I), again at elevated temperature, although not involving free-radicals in this case (Saegusa et al., 1970). The investigations reported here found that the product of reaction was the 8-amino nucleoside or nucleotide depending on the starting material, and irrespective of the dithiol used. Neither metal catalysts nor high temperatures were required for rapid, high yield reaction.

A kinetic analysis of the reaction was undertaken in the hope that the results could be interpreted in mechanistic terms. Under comparable conditions, dithiols were found to give generally much higher rates of reduction than a series of monothiols (see Table 2.2). The reaction obeyed second-order kinetics and a typical rate plot is shown in Figure 2.12. The higher rate of reduction by dithiols appeared to have its origin in a neighbouring group effect since in the experiments with monothiols at double the concentration (i.e. equivalent numbers of -SH groups) the rate was extremely low. When the concentration of 2-mercaptoethanol (monothiol) was increased by 50-fold, a comparable rate of reduction to that of DTT (at 0.26 mM) was observed (Figure 2.13), and kinetics of reduction were pseudo-first order. This further implicates the neighbouring thiol groups of the dithiols as important moieties in the reaction mechanism. It should be noted also from Table 2.2 that the number of intervening carbon atoms between the two thiol moieties appears to bear a relation to the relative rates of reduction of the azides.

These observations lead to the conclusion that reduction of the azide to amine with evolution of nitrogen is accompanied by oxidation of the thiols to the disulphide (cf. Saegusa et al., 1970); in the case of the dithiols, a facile ring closure could be effected to produce the cyclic disulphides. In the case of

Table 2.2

Relative reaction rates of thiols with z^8AMP

Thiol	Relative rate of reaction
Dithiothreitol	100
Dithioerythreitol	100
1, 3-propane dithiol	269
2, 3-dimercaptopropanol	6
1, 2-ethanedithiol	2.5
L-cysteine	2
Thiophenol	0
2-mercaptoethanol	0

To 3 ml of 1 mM dithiol (2 mM monothiol) in methanol at 25° was added triethylamine (50 µl) followed by z^8AMP (triethylammonium salt, 25 µl, 12.8 mM in methanol. The reaction was monitored at 300 nm as described in Section 2.2.2. Under the conditions of this experiment, reaction of thiophenol or 2-mercaptoethanol was not detectable; the second order rate constant for reaction of DTT was $1.15\ M^{-1}s^{-1}$.

Figure 2.12

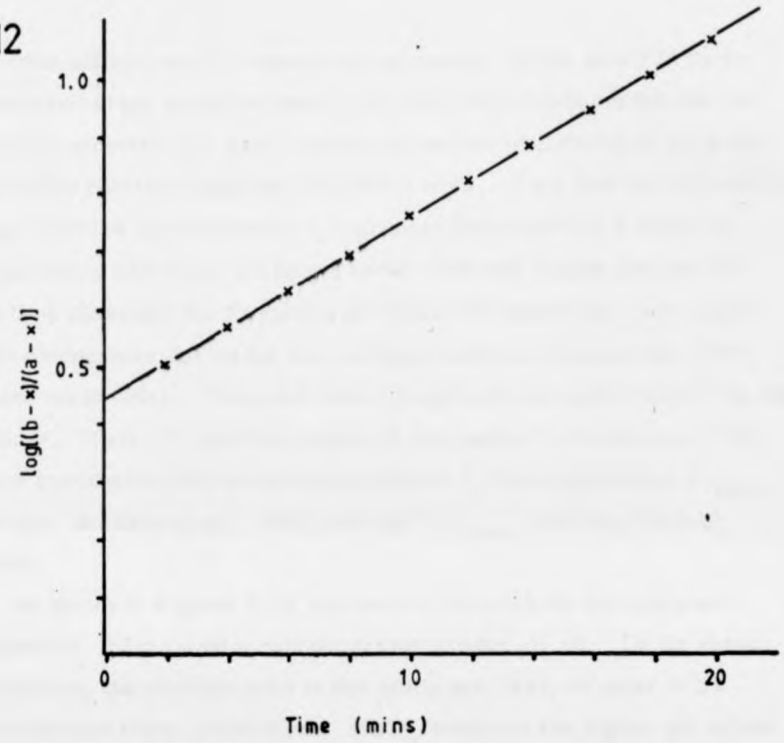
Second order kinetic plot for the reaction of z^8AMP (9.38×10^{-5} M) with dithiothreitol (2.58×10^{-4} M) in 0.1 M borate-phosphate, pH 8.9

Second order rate constant $k_2 = 7.41\ M^{-1}s^{-1}$

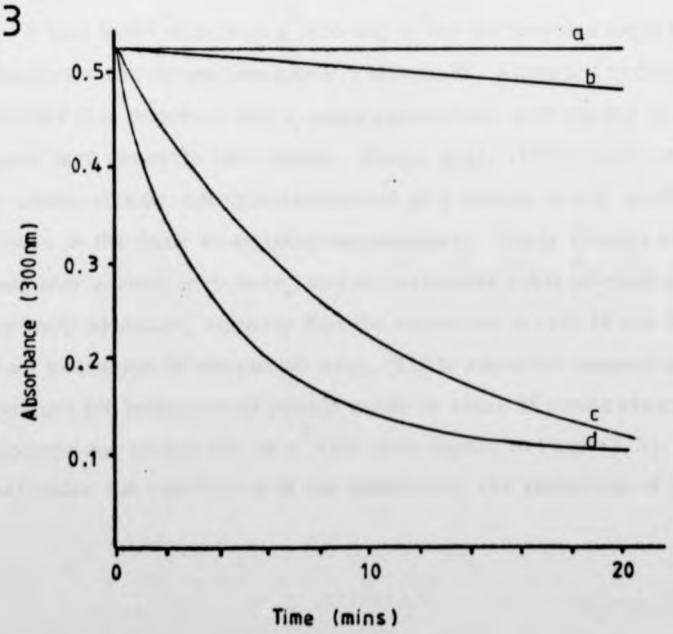
Figure 2.13

The reaction between 2-mercaptoethanol and z^8AMP incubations, as described in Table 2.3, were performed in 0.1 M glycine-NaCl-NaOH buffer, pH 9.1, with (a) 0.52 mM, (b) 10 mM, (c) 100 mM 2-mercaptoethanol. Incubation with 0.26 mM dithiothreitol. (d) is shown for comparison.

2.12


1.0
0.5
log[(b - x)/(a - x)]
0
10
20
Time (mins)

2.13


a
b
c
d
0.5
0.4
0.3
0.2
0.1
Absorbance (300nm)
0
10
20
Time (mins)

1,2-ethanedithiol and 2,3-dimercaptopropanol, highly strained four-membered rings would be found; the rates might well reflect the low stability expected for such a structure and the possibility of an intermolecular reaction might be considered here. Five and six-membered rings would be expected from 1,3-propanedithiol and DTT (or DTE) respectively and these are known to be relatively stable compounds. We have observed the formation of weakly UV absorbing, non-nucleotidic components during the ion-exchange column purification of the amino nucleotides. These non-ionic compounds are not retained by the column. Their UV spectra (Figure 2.14) appear to be identical with those reported for the cyclic disulphides of 1,3-propanedithiol (λ_{max} 330 nm; Barltrop et al., 1954) and DTT (λ_{max} 283 nm; Cleland, 1964).

As shown in Figure 2.15, the rate of reduction is markedly pH-dependent, the maximum rate occurring around pH 10. Under these conditions, the dithiols used in this study are likely to exist in the monothiolate form (Antikainen, 1962), whilst at the higher pH values the dithiolate predominates. However, the reduction in rate at high pH is not likely a consequence of the second ionisation (see discussion of mechanism below).

It was found that both z^8Ado and z^8Ino nucleotides were highly reactive under these conditions (Table 2.3). Attempts to discover whether this reaction was a more general one with azides of different types have recently been made. Staros et al. (1978) have reported that an identical base-catalysed reduction of a series of aryl azides by DTT occurs in the dark at ambient temperature. Their results are in excellent accord with ours, and in particular their pH-rate profile is virtually identical, showing that the reduction in rate is not likely due to an ionisation in the purine ring. Their reported second-order rate constant for reduction of phenyl azide is some 25 times lower than that reported for reduction of z^8AMP (see legend to Table 2.3). We found that under the conditions of our reactions, the reduction of phenyl

Figure 2.14

Ultra-violet spectra of non-nucleotidic products of reaction of z^8AMP with (A) 1,3-propanedithiol and (B) dithiothreitol

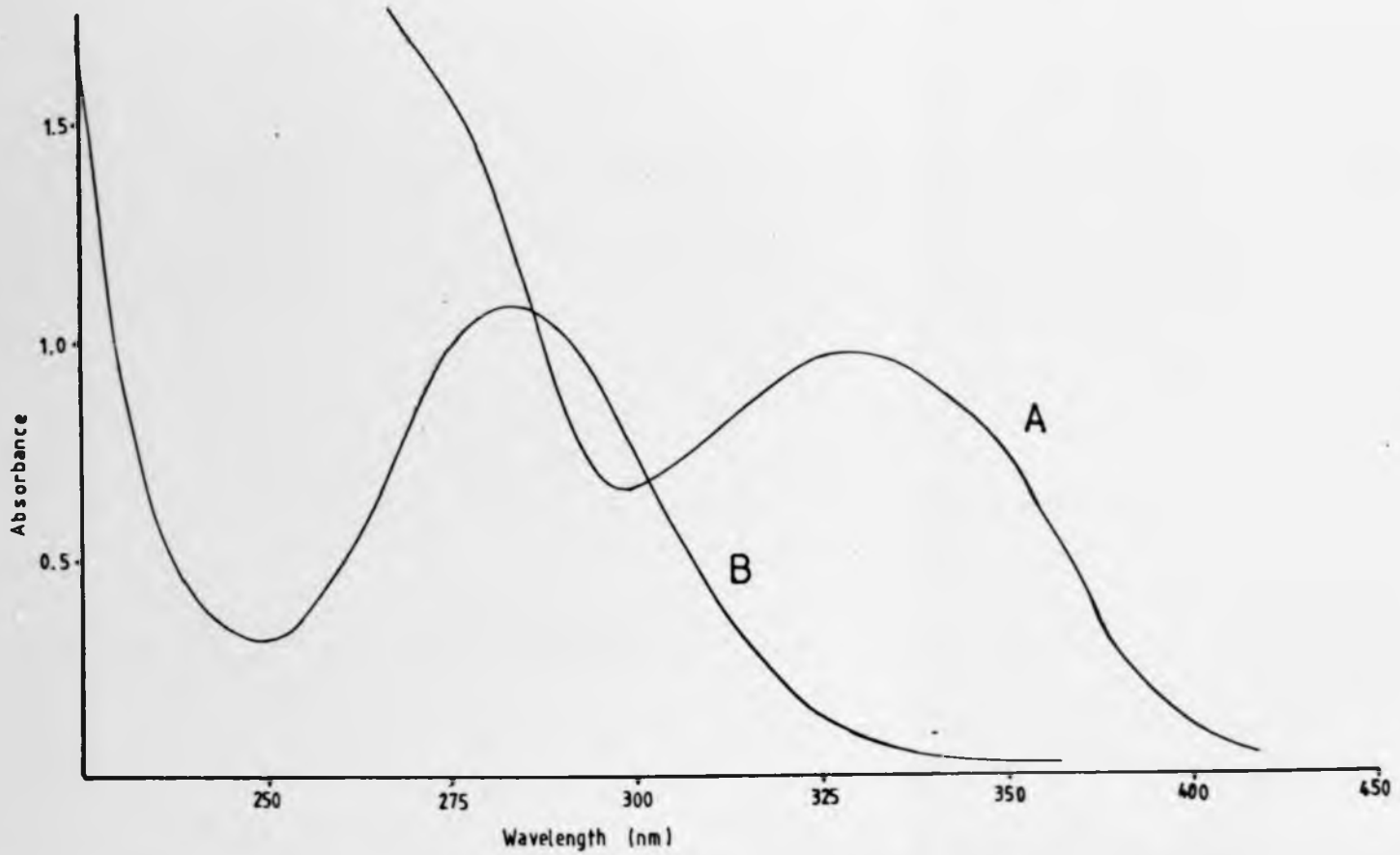

1.5
1.0
0.5
Absorbance
A
B
250
275
300
325
350
400
450
Wavelength (nm)

Figure 2.15

Effect of pH on the relative rate of reaction of z^8AMP with dithiothreitol. Reactions were performed in 0.1 M buffers and monitored by decrease in absorbance at 300 nm, using z^8AMP (9.35×10^{-5} M) and dithiothreitol (2.58×10^{-5} M). Buffers used were: pH 5-7, citrate-phosphate; pH 8-9 borate-phosphate; pH 10-12 glycine-NaCl-NaOH

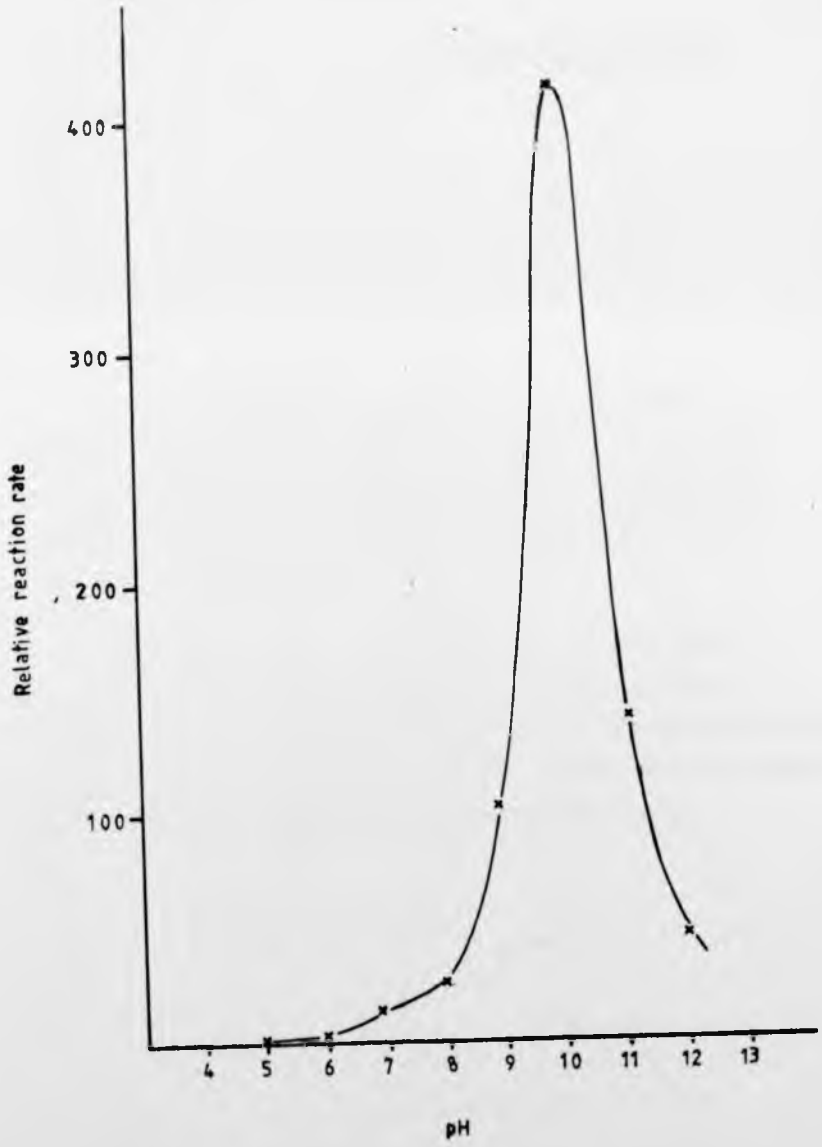

Relative reaction rate
400
300
200
100
4
5
6
7
8
9
10
11
12
13
pH

Table 2.3

Relative rates of reaction of dithiothreitol with azides

Substrate	Relative rate of reaction
z^8 Ado	464
z^8 AMP	100
z^8 ADP	83
z^8 IDP	27
phenyl azide	0

(a) To 0.1 M borate-phosphate buffer (3 ml, pH 8.9) at 25° was added 10 mM DTT (80 µl) and 0.5-0.7 $O.D._{300}$ unit of nucleoside or nucleotide. A typical second order plot can be derived under these conditions (see Figure 2.12); the raye constant found for z^8 AMP is 7.41 $M^{-1}s^{-1}$ ≡ 100 (above).

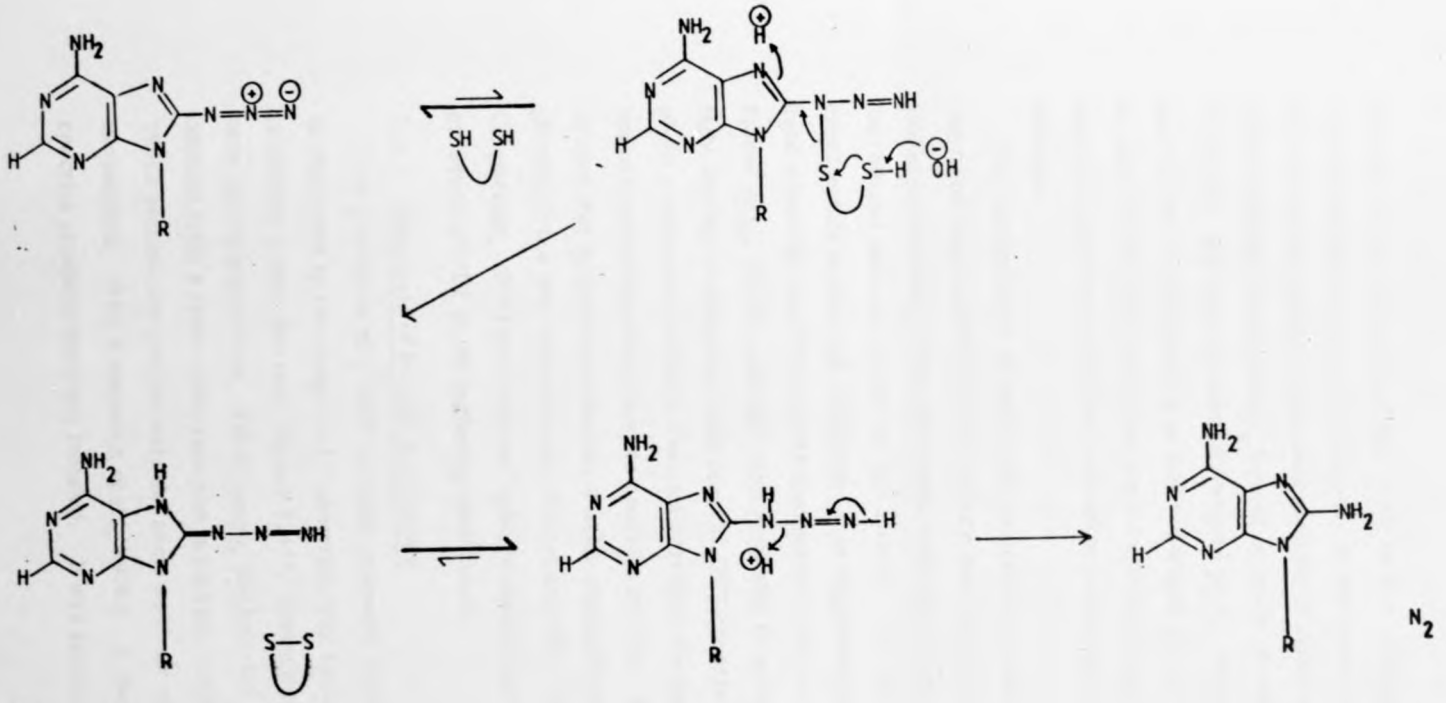
(b) Phenyl azide (0.15 mM) was circulated in borate-phosphate/methanol (1:1 v/v) containing DTT (1 mM). No reaction could be observed under these conditions (but see text). In the same solvent system, the relative rate of z^8 AMP reaction was 30. Addition of diphenyl picryl hydrazyl up to 40 mole % had no effect on the reaction rate.

azide to aniline did not occur. Only when a large excess of DTT was added, and the solution made alkaline by addition of sodium hydroxide, did we observe the production of aniline, and then only after overnight incubation. Extreme difficulty was experienced in solubilising phenyl azide in the aqueous buffers used, and it was necessary to use mixtures with methanol to achieve conditions where the reaction could be studied. Conversely, Staros et al. (1978) report their studies on 2 mM phenyl azide in 0.1 M salt buffers. The reason for this discrepancy is unclear at present. Further work by this group (Bayley et al., 1978) has confirmed that 1,3-propanedithiol is the most effective reducing agent of this type known so far, and will even reduce alkyl azides in the presence of triethylamine when extended reaction times and higher temperatures are used. Such situations are similar to the early studies by Shingaki (1963) when a free radical mechanism was implied. In the experiments reported here, addition of the radical scavenger, diphenyl picryl hydrazyl had no effect on the reduction rate, and a free-radical mechanism is discounted. In agreement with our findings (for DTT), Bayley et al. (1978) reported that 1,4-butane-dithiol reacted more slowly in these experiments than 1,3-propane-dithiol, so that a postulated ring-closure mechanism appears to be common to these reactions.

A plausible mechanism is proposed in Figure 2.16 which appears to account for both the known reaction products and the observed kinetics/pH-dependence. Attack of the monothiolate ion on the α-nitrogen in a rapid, reversible reaction is followed by slow rate-determining ring-closure of the dithiol, giving the sulphenylamide and ultimately loss of nitrogen and generation of the amine. Such a mechanism is consistent with the observed low rate of reduction by monothiols, since intermolecular attack on the monothiol-azide adduct would be expected to be much slower than the intramolecular reaction. Presumably, the initial equilibria lie in favour of the reactants so that a large increase in the concentration of monothiol would accelerate the reaction rate. Increase of pH would

Figure 2.16

A possible mechanism for the reaction between 8-azido nucleoside and dithiols



R = ribose or ribose 5'- phosphate

probably lead to ionisation of the γ-NH or N-7 of the purine, and competition with disulphide formation. In the formally similar reaction involving Cu(I)-catalysed reduction of azides by monothiols, sulphenylamide intermediates, formed by attack of -SH on the α-nitrogen, are readily isolated (Saegusa et al., 1970). Other mechanisms involving attack at the γ-nitrogen can be visualised, but the fact that the fully protonated dithiol shows no reaction argues against a concerted reduction followed by prototropy and loss of nitrogen.

The identification of this facile reduction of azides by dithiols has important implications for photoaffinity labelling experiment. In many biological systems, thiols are useful adducts for the maintenance of the reduced state necessary for full activity. For photoaffinity labelling experiments in this type of system, it is suggested that monothiols be used whenever possible to avoid destruction of the azido photo-label. Staros et al. (1978) make the valid point that in systems where very tight binding of the photo-label occurs, DTT could be added prior to photo-activation in order to chemically reduce the non-specifically bound azide which often produces spurious results. It is of some interest to note that spontaneous reaction of azido photoaffinity labels prior to photolysis has now been reported (Wetzel and Söll, 1977; Nicholson and Cooperman, 1978) and it seems highly probable that this was due to the presence of DTT in the buffering media used.

2.3.3 Photolysis of 8-azido nucleotides

The photolysis of z^8AMP in water proceeds rapidly and smoothly, as disclosed by the change in UV spectrum with time, and the number of isosbestic points obtained (Figure 2.17). Under the conditions used here (pyrex glass filter, 100 W lamp), the half-life of photolysis derived from a first-order rate plot is 62.9 seconds (Figure 2.18). These results are qualitatively the same as those observed previously (Koberstein, 1976; Koberstein et al., 1976). In these earlier studies, reaction products were not isolated. It was assumed that the major

Figure 2.17

Change in ultra-violet spectrum of z^8AMP on photolysis in water. Spectra were recorded with the pyrex filter in place at

(a) 0" (b) 30" (c) 60" (d) 90" (e) 120" and (f) 150" at a concentration of 4.45×10^{-4} M

The inset shows the transmission characteristics of pyrex (———) and pyrex/soda glass combination (------) filters.

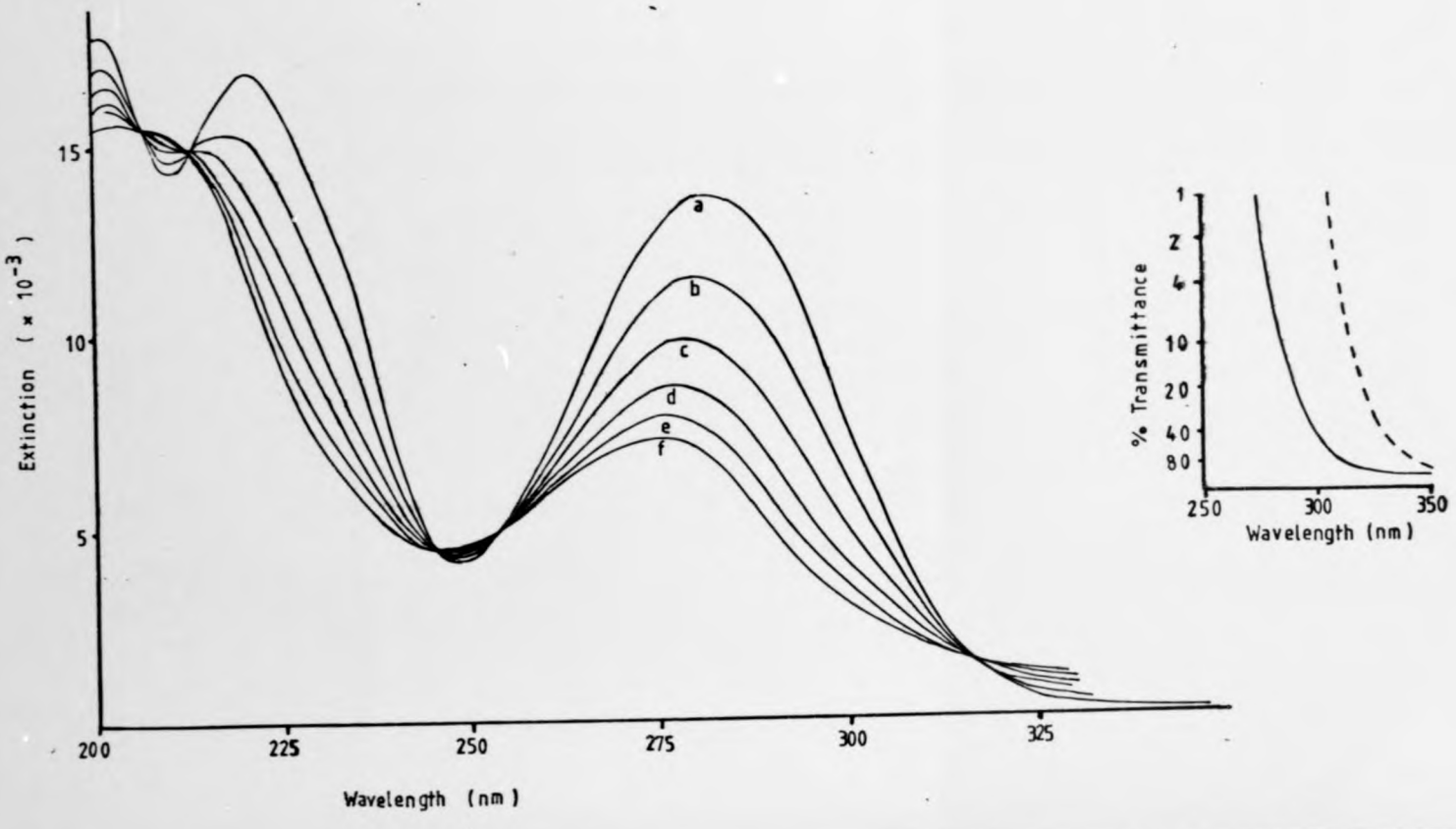
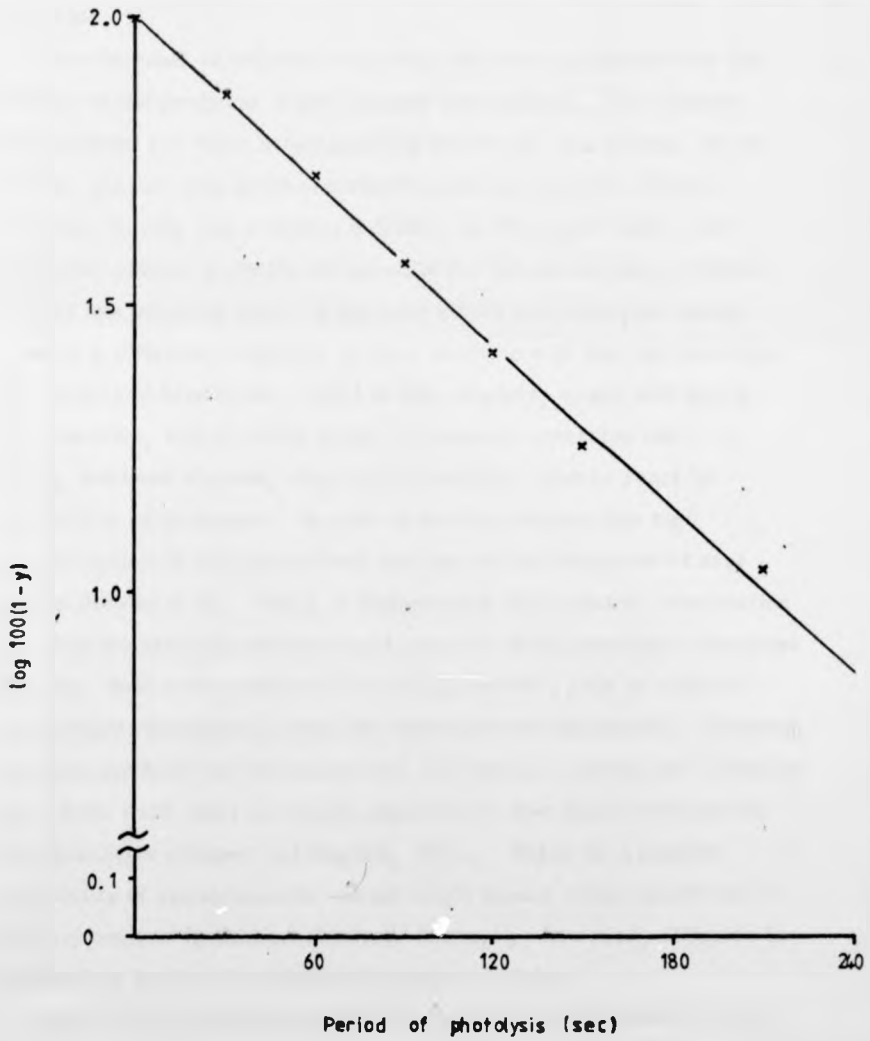

Extinction (× 10⁻³)
15
10
5
200
225
250
275
300
325
Wavelength (nm)
a
b
c
d
e
f
% Transmittance
1
2
4
10
20
40
80
250
300
350
Wavelength (nm)

Figure 2.18

First order kinetic plot for z^8AMP photolysis. Conditions were as described in Figure 2.17. The first order rate constant $k_1 = 1.1 \times 10^{-2} s^{-1}$ and the half-life $t_{\frac{1}{2}} = 62.9$ seconds. The quantity (1-y) (where y = extent of reaction ($o \leq y \leq 1$)) is equivalent to (a-x) and is derived from optical density data at 270 nm


2.0
1.5
1.0
0.1
0
log 100(1 - y)
60
120
180
240
Period of photolysis (sec)

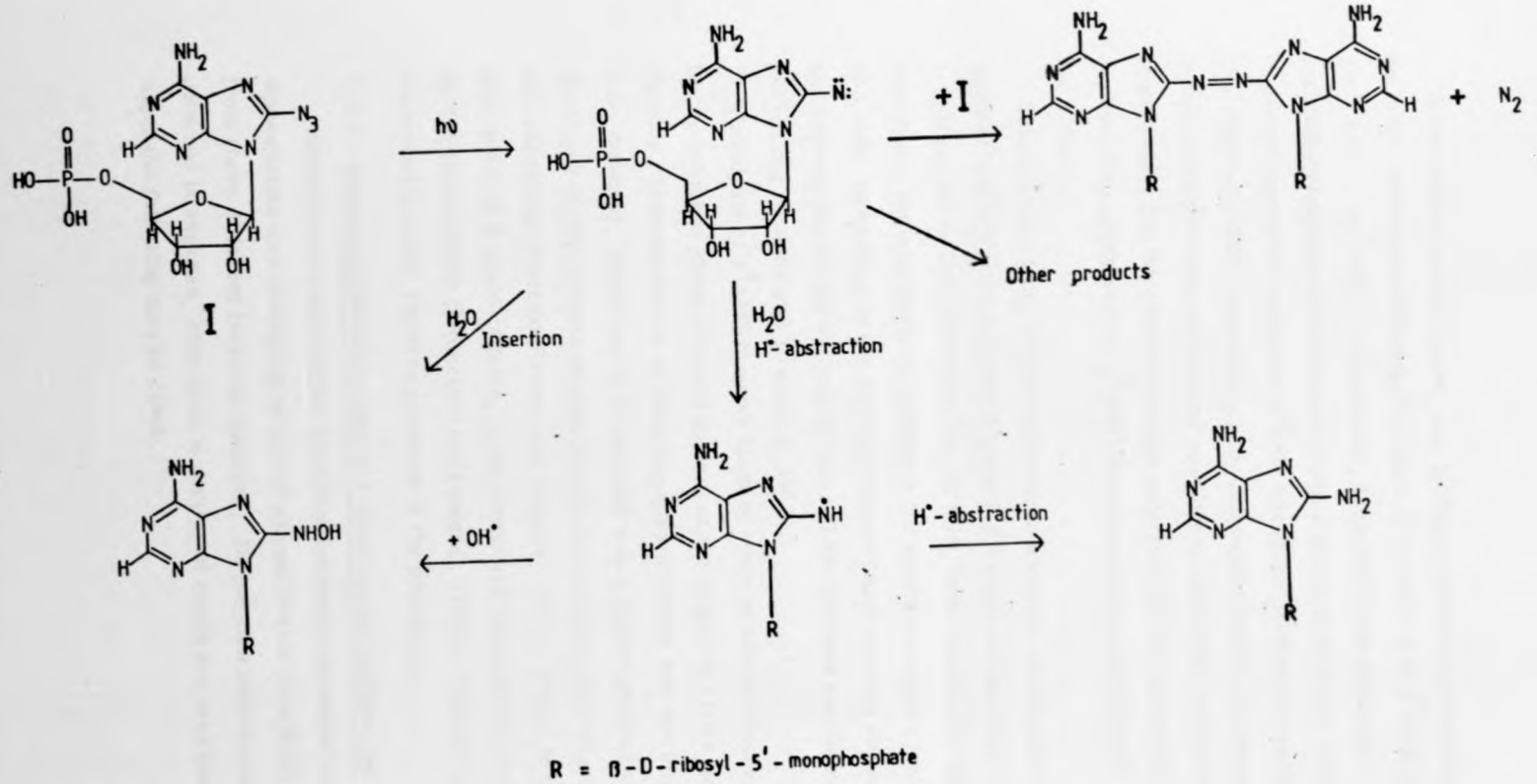
product was 8-hydroxylamino-AMP found by insertion of the putative nitrene into water. As evidence, it was observed that the product of reaction between br^{8}AMP and hydroxylamine displayed a UV spectrum very close to that observed for the final photolysis product of z^{8}AMP.

For a number of reasons it was felt that this explanation for the identity of the probable major product was unlikely. The nitrene intermediate can exist in two possible electronic spin states. In the singlet, the two lone pairs of electrons are spin-paired in filled orbitals, leaving one p-orbital unfilled. In the triplet state, one molecular orbital is doubly-filled while the two remaining p-orbitals contain one electron each. It has been shown that these two states possess a different chemistry in their reacting with the surroundings (Liwowski and Mattingley, 1965) in that singlets, which are strong electrophiles, add to double bonds and undergo insertion into C-H bonds, whereas triplets, which are biradicals, mainly react by abstraction of hydrogen. In view of the observation that high concentrations of triplet nitrenes are formed on photolysis of aryl azides (Reiser et al., 1968), it seems more likely that H$^{\cdot}$-abstraction would be the primary mechanism of reaction of the generated adenosine nitrene. Such a reaction in water would generate a pair of radicals which might recombine to form the hydroxylamine derivative. However, radicals produced by this mechanism are not spin-paired, and diffusion away from each other is usually much faster than spin-reversal and recombination (Reiser and Wagner, 1971). There is a certain probability of recombination, so one might expect small quantities of hydroxylamines by this mechanism. Normally, however, diffusion is followed by further H$^{\cdot}$-abstraction to give an amine.

Insertion of aryl nitrenes into O-H bonds does not appear to be a common reaction, although known for carbonyl nitrenes. In a study of the photolysis of 8-azido-caffeine in a series of alcohols, there was no observation of O-H insertion, the major products being due to abstraction of hydrogen (Sutherland and Pickard, 1974) to yield 8-amino compounds.

Figure 2.19

Reaction products in the photolysis of z^8AMP under aqueous conditions


hν
+ I
+ N2
Other products
H2O
Insertion
H2O
H•- abstraction
+ OH•
H•- abstraction
R = β-D-ribosyl-5'-monophosphate

In the present investigation, convincing evidence was obtained that the major low molecular weight product of photolysis of z^8AMP in water was n^8AMP. This product, isolated by ion-exchange chromatography possessed characteristics identical in every respect to the hydrogenation product of z^8AMP and to the product of reaction of z^8AMP with DTT (see above). On dephosphorylation, all three compounds gave spots of the same mobility on silica tlc, and most importantly, the low resolution mass spectrum of the nucleoside derived from photolysis of z^8AMP corresponded to that expected for n^8Ado.

Thus evidence from UV spectra alone can lead to ambiguous results (comparison of Figure 2.9 with Figure 2.8 shows the homology). 8-Hydroxylamino-adenosine has not, in fact, been reported in the literature, but it is likely to possess a UV spectrum almost identical to n^8Ado, by analogy to the correspondence found between the amino and hydroxylamino derivatives of Guo and Ino (Holmes and Robins, 1965; Long, Robins and Townsend, 1967).

Photolysis of z^8AMP in water leads to other products which are highly coloured (deep red) and remain at the origin on silica tlc. Further characterisation of these complex products was not attempted. It is, however, likely that 8, 8'-azo AMP was a major product since the attack of aryl nitrene on precursor azide to yield azo compounds is a ubiquitous reaction (Reiser and Wagner, 1971). Thus, in the photolysis of 8-azido-caffeine, a red compound isolated was found to be 8, 8'-azocaffeine (Sutherland and Pickard, 1974). Figure 2.19 shows the probable major reaction products of the photolysis.

2.3.4 Homopolyribonucleotides of 8-azido purine nucleotides

Homopolymerisation of the 8-azido substituted adenosine and inosine diphosphates was attempted by use of polynucleotide phosphorylase from three different bacterial sources. No polymerisation was detected in any case, even when a variety of conditions was used, amongst which the following may be cited:

(i) Variation of the metal ion concentration from 0.5 mM to 5 mM

(ii) Both Mg^{2+} and Mn^{2+} used

(iii) z^{8}NDP concentration varied between 1 mM and 10 mM

(iv) Addition of sodium chloride up to 0.2 M

(v) Temperature of incubation varied from 37° C to 65°C

(vi) Incubations with and without oligonucleotide primers $(Ap)_3A$ and $(Up)_2U$ as well as poly A_n and poly I_n.

Of the three enzymes used, one was a commercial product from M. luteus, whilst samples from B. stearothermophilus and E. coli were prepared in the laboratory by techniques as described (Wood and Hutchinson, 1976). In each case, the enzyme reaction was monitored by release of inorganic phosphate and occasionally the products of incubation were subjected to gel filtration. No high molecular weight product was ever isolated, even in those cases where the phosphate assay appeared to show some reaction. This was shown to be due to a very low level of phosphatase activity present in some preparations of the enzyme. Use of the thermophilic enzyme from B. stearothermophilus allowed high temperatures of incubation to be maintained, but in analogy to Wood and Hutchinson (1976), not even a small degree of reaction could be detected.

No report has yet appeared on the ability of such enzymes to polymerise syn nucleotides, and the present investigation endorses this. Numerous attempts have been made to produce syn polyribonucleotides by enzymatic means. Thus Ikehara et al. (1969) and Kapuler et al. (1970) found that 8-substitution in purines and 6-substitution in pyrimidines led to total resistance to polymerisation by polynucleotide phosphorylase, and it was suggested by the latter workers that the enzyme would require a syn $\rightleftharpoons$ anti equilibrium to enable polymerisation to be effected. This point seems to have been confirmed in other work. 4-Thiouridine diphosphate is a syn conformer in the crystalline state and could not be homopolymerised by the M. luteus PNPase (Scheit and Gaertner, 1969). However, use of the E. coli enzyme allowed

$(s^4U)_n$ to be synthesised (Simuth et al., 1970). The polymer displayed normal complex formation with A_n and in every respect behaved as if the anti conformation had been adopted in the polymer. A similar investigation with the syn nucleoside formycin (an analogue of adenosine) showed that homopolymerisation proceeded very slowly (Ward et al. (1969), and that the poly formycin produced displayed properties that could be interpreted only in the light of syn $\rightleftharpoons$ anti equilibria (Ward and Reich, 1968). Thus, normal complex formation with U_n occurred (anti behaviour), but in the single stranded form anomalous spectroscopic and nuclease digestion properties suggested that an all-syn conformation could be adopted. In copolymers with A, it was concluded that a mixture of syn and anti forms co-existed. Some 8-substituted purine nucleotides have been homopolymerised in good yield. However, it seems likely that the anti form is that naturally occurring or at least easily attainable, since they involved the small 8-amino (Howard et al., 1972) and 8-keto (Folayan and Hutchinson, 1977) groups. Both polymers exhibited normal complex formation with U_n characteristic of anti polynucleotides.

There has, however, been one isolated report of the enzymatic synthesis of an all-syn polynucleotide, namely $(br^8A)_n$ (Howard et al., 1975). Using E. coli PNPase, Mn^{2+} ions and $(Ip)_3I$ primer, the polymer was obtained in 57% yield and displayed physical properties quite unlike those of previously synthesised polymers. Thus, under normal solution conditions, a parallel, double stranded helical structure existed, with efficient hydrogen-bonding between opposing br^8A residues. No complex formation with U_n was found to occur, nor could A_n substitute for one of the br^8A strands in the native duplex (Govil et al., 1977). The authors found that in the absence of primer, no polymerisation of br^8ADP occurred, and attributed this to lack of chain initiation by the enzyme in the presence of syn nucleotides. Chain elongation, however, appeared to occur readily (albeit at a reduced rate relative to ADP), and it was suggested that the syn $\rightleftharpoons$ anti equilibrium previously postulated appeared unnecessarily complex to explain the catalysis observed.

Aside from this isolated report of enzymatic synthesis, it has been found possible to introduce by chemical means bulky groups into the C-8 position of pre-formed polynucleotides. Michelson et al. (1970) brominated G_n and reported that the $(br^8G)_n$ obtained displayed very unusual properties somewhat akin to those observed with $(br^8A)_n$ (above). Notably, unusual ORD/CD spectra and failure to form complexes with C_n or $(dC)_n$ was observed. Non-cooperative thermal transitions indicated a random coil conformation in contrast to $(br^8A)_n$.

It seems probable that the reported $(br^8A)_n$ could be transformed chemically to $(z^8A)_n$. The substitution of bromide for azide does, however, have to be accomplished in non-aqueous media, but methods are available for efficient solubilization of RNA in organic solvents using the cetyl trimethylammonium ion as a replacement counterion (Aubel-Sadron et al., 1961). The recovery of these RNA species in a water-soluble form has been reported to occur without any loss in physical characteristics or biological properties (Ebel et al., 1965) so in principle this method could provide a route to $(z^8A)_n$ which might well be explored in future studies.

On consideration of the reported properties of those polynucleotides already synthesised with bulky groups in the purine 8-position, it seems likely that 8-azido substituted polynucleotides would display rather unusual structural characteristics. In particular, double-stranded formation with complementary polynucleotides would be unlikely to occur. Since normal RNA duplex formation appears to be a prime requirement for the process of interferon induction by polynucleotides, the synthesis of the homopolymers was not pursued further in the present investigation. On the other hand, the synthesis of co-polymers containing low percentages of 8-substituted purine base has been reported to occur readily with PNPase (Ikehara et al., 1969). Furthermore, such copolymers may well display relatively normal complex formation and would then be useful in the study of interferon induction (see Chapter 4).

2.3.5 Co-polymerisation Studies

In contrast to the results obtained for homopolymerisation, co-polymerisation of the modified 8-azido purine nucleotides with their

unsubstituted congeners led to slow but efficient synthesis of high molecular weight polymers. Figure 2.20 shows a typical reaction progress curve, similar in qualitative terms to the copolymerisation of br^{8}ADP with ADP (Ikehara et al., 1969). Yields estimated by phosphate release were always in excess of 45%. UV spectra of the purified copolymers are shown in Figure 2.21 and 2.22 for $(z^8A, A)_n$ and $(z^8I, I)_n$ respectively compared to their unsubstituted homopolymers. It will be noticed that both display a long wavelength shoulder, extending out beyond 300 nm, due to the 8-azido purine nucleotide content. This absorption makes both polymers suitable for photolysis at these long wavelengths where damage to biological systems should be minimised. The effect of such photolysis is shown in the UV spectra of Figure 2.23; the soda glass filters used during photolysis are as shown in Figure 2.17 (inset). It will be seen that destruction of the azido moiety takes place readily under these conditions and allows the conclusion to be drawn that such polymers could be usefully employed in photoaffinity labelling experiments.

Copolymers of both the adenosine and inosine series were synthesised with a variety of input base ratios, and Table 2.4 shows the characterisation of some of these polymers. The sedimentation values of those polymers containing higher proportions of the 8-substituted base are smaller, implying that under conditions where the enzyme is forced to accept the syn nucleotide with greater frequency, efficiency of chain elongation is lower. It is known that PNPase will terminate polymers prematurely when it incorporates atypical substrates into a chain. Thus dADP, when incorporated into a polymer, will add one or possibly two successive residues before chain termination (Bon et al., 1970). Similarly, substrates with bulky steric requirements at the 2' position, e.g. 2'-O-(α-methoxyethyl), are only added in a "single addition" reaction (Bennet et al., 1973). It was thus of interest to determine the 3'-terminal nucleotide in a typical sample of polymer. Figure 2.24 shows the results of a 3'-terminal labelling experiment which demonstrates, under the limits of detection of the experiment, that the only 3'-terminal residue in a sample of $(z^8A, A)_n$ is that of z^8A. It appears then, that not only is

Figure 2.20

The polymerisation of inosine nucleotides as determined by inorganic phosphate release. Conditions were tris-HCl, pH 9.0 (50 mM), Mn^{2+} (3 mM), E. coli PNPase (0.75 U) and either IDP (10 mM) (X – X – X) or IDP (8 mM) plus z^8IDP (2 mM) (□–□–□) in a volume of 0.5 ml. Incubation was at 45°, and the extent of polymerisation was similar at the 18 hour time-point

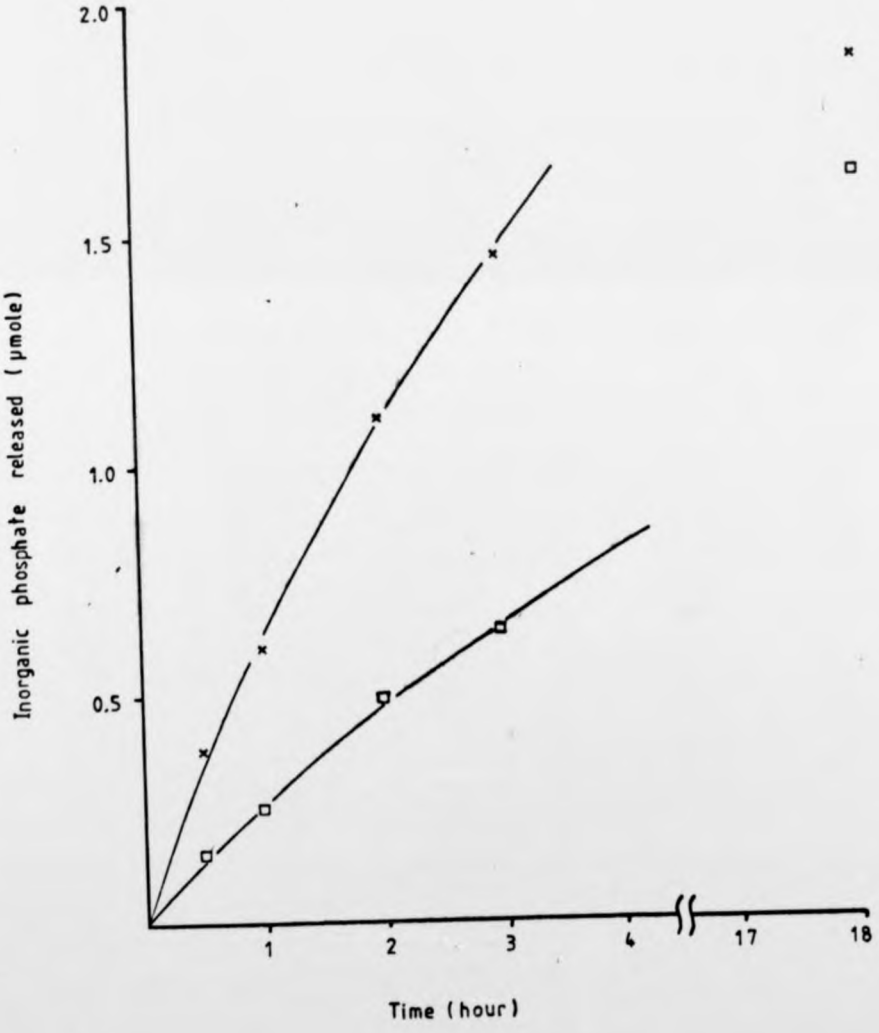

2.0
1.5
1.0
0.5
Inorganic phosphate released (μmole)
1
2
3
4
17
18
Time (hour)

Figure 2.21

Ultraviolet spectra of $(z^8A, A)_n$ (———) (z^8Ado content = 12.5%) and A_n (------) at the same Abs.$_{\lambda max}$

Spectra performed in 0.01 M sodium cacodylate, 0.1 M sodium chloride, pH 7.0

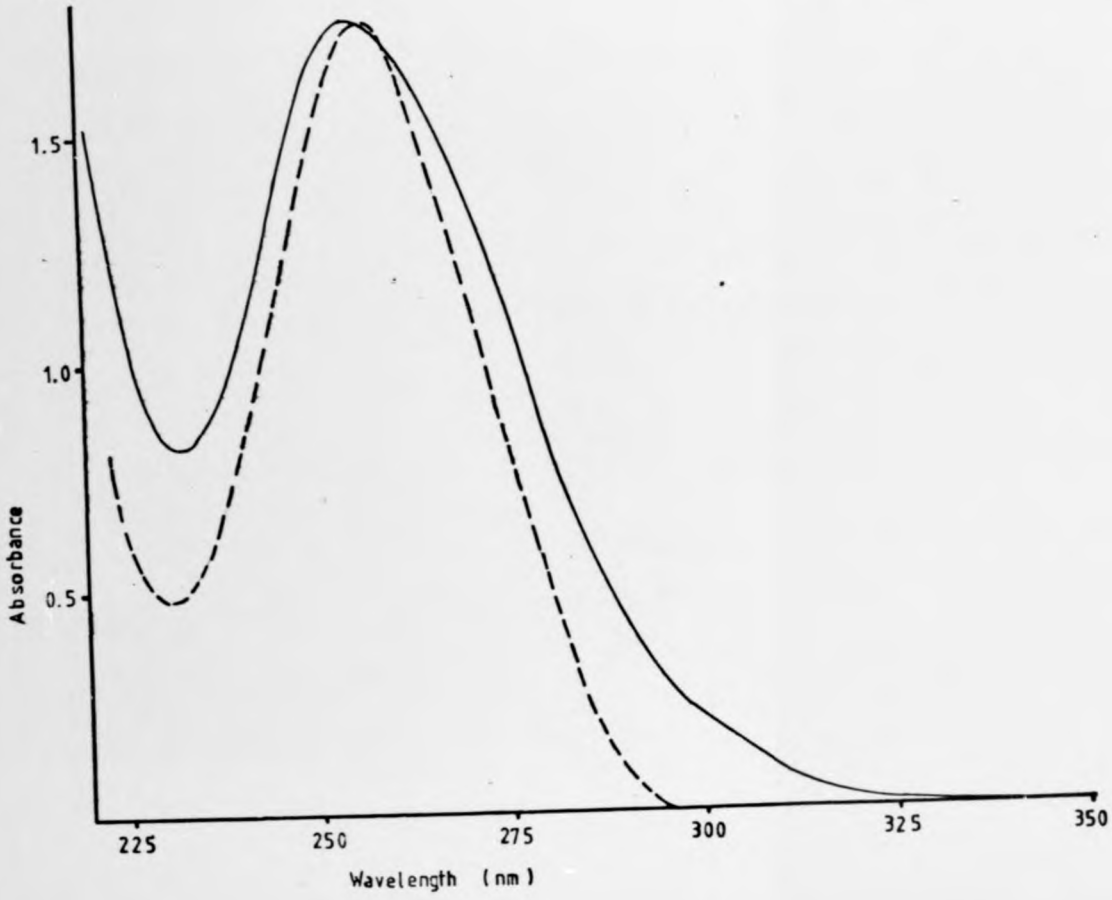

1.5
1.0
0.5
Absorbance
225
250
275
300
325
350
Wavelength (nm)

Figure 2.22

Ultra-violet spectra of $(z^8I, I)_n$ (------) (z^8Ino content = 2.5%) and I_n (——) at the same Abs.$_{\lambda max}$

Spectra performed in 0.16 M sodium phosphate, pH 7.0

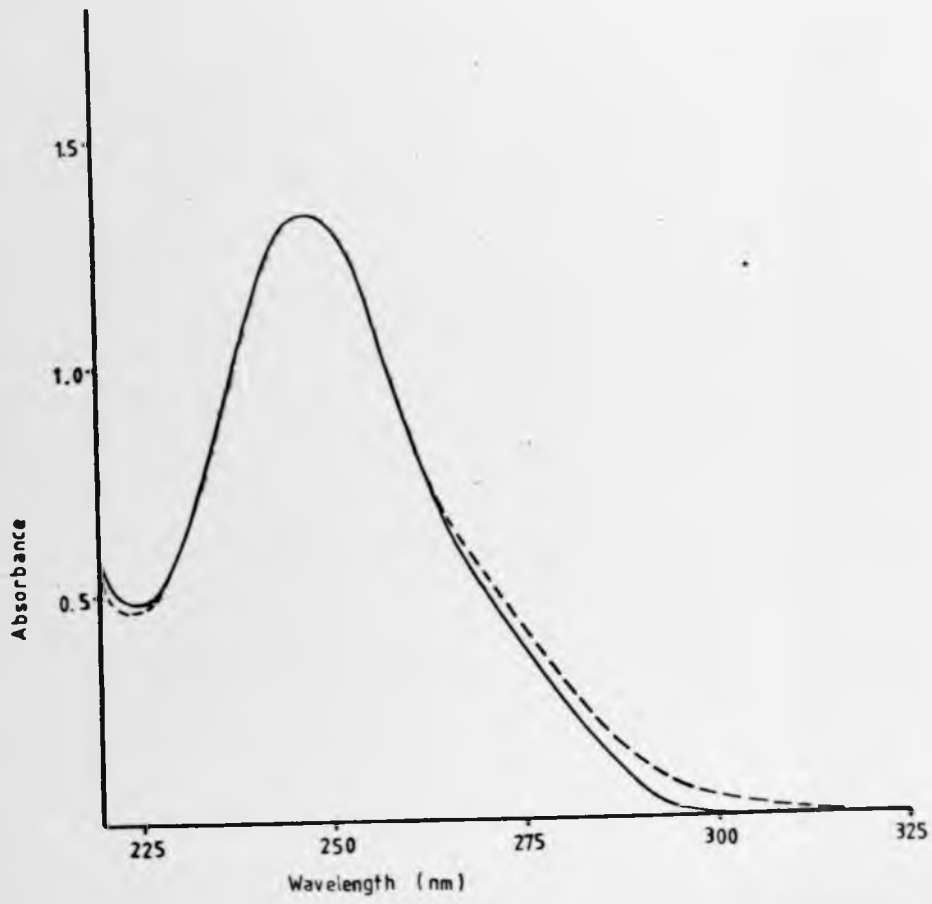

1.5
1.0
0.5
Absorbance
225
250
275
300
325
Wavelength (nm)

Figure 2.23

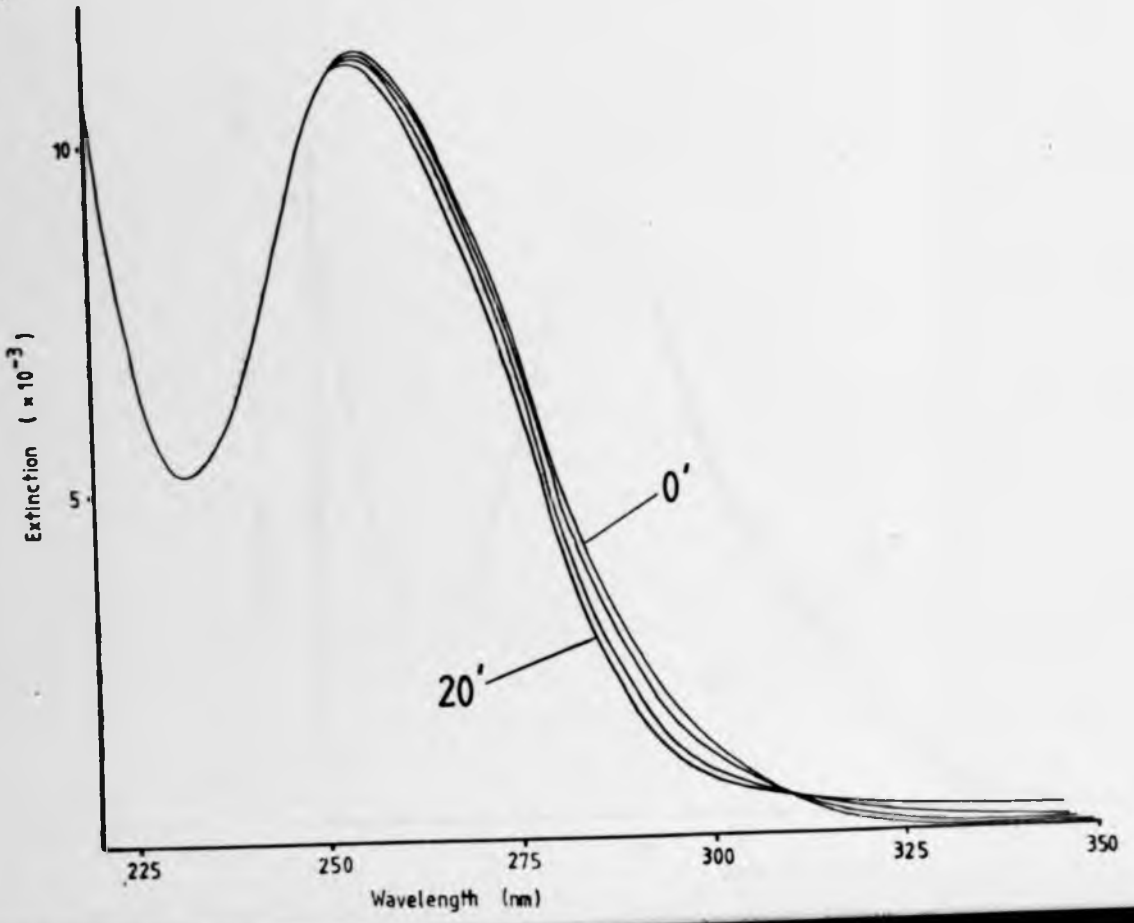
Change in ultra-violet spectrum of copolynucleotides on photolysis under aqueous conditions

(a) $(z^8A, A)_n$ (z^8Ado = 12.5%) at 0', 3', 10' and 20'

(b) $(z^8I, I)_n$ (z^8Ino = 2.5%) at 0', 2', 7' and 27'

The pyrex/soda glass filter combination was used (see Figure 2.17) and the energy flux falling on the solutions was 2×10^4 erg/sec/mm^2

a)


10
5
Extinction ($\times 10^{-3}$)
0'
20'
225
250
275
300
325
350
Wavelength (nm)

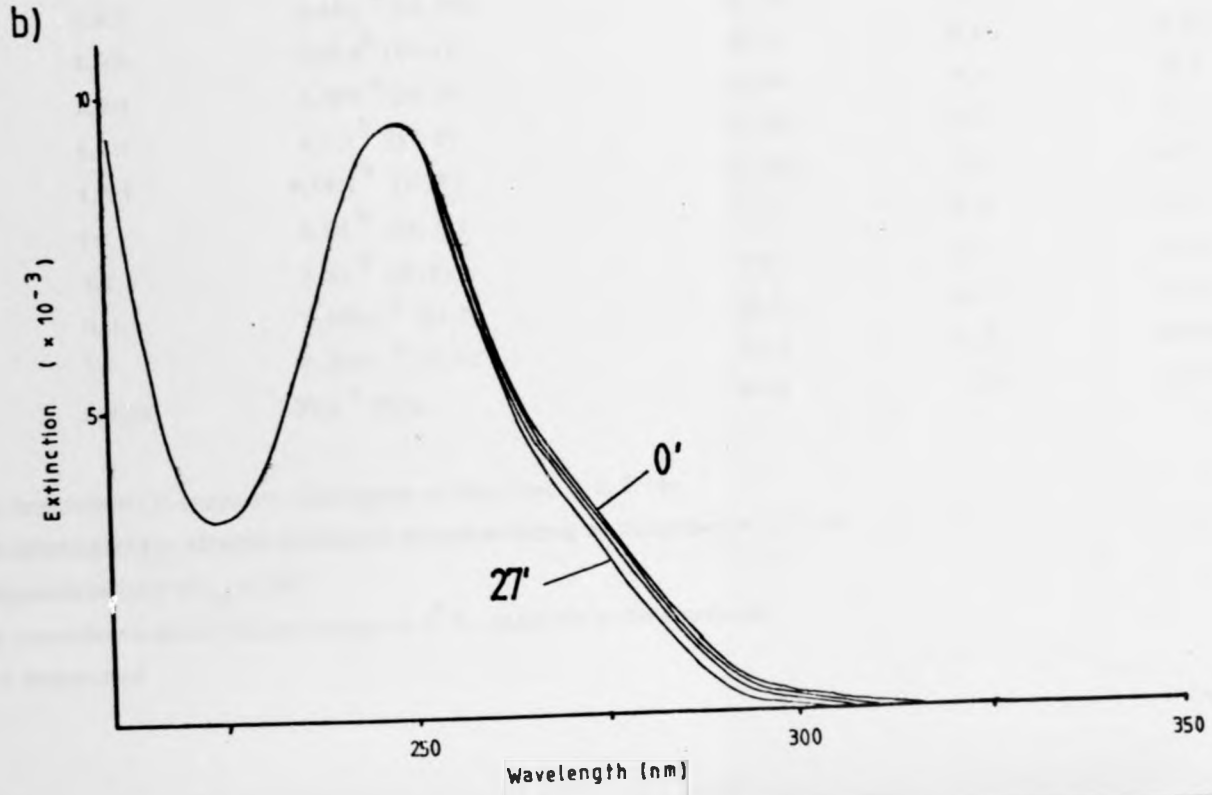

b)
10
5
Extinction (× 10⁻³)
0'
27'
250
300
350
Wavelength (nm)

Table 2.4 Some properties of samples of synthetic azido copolynucleotides

Copolymer	Ratio of NDP:z^8NDP in polymerisation mixture	Ratio of nucleosides (N:z^8N) in copolymer	Extinction coefficient (ε_p)	Sedimentation coefficients (S)	Hyperchromicity [c] %
$(z^8A, A)_n$	3.2:1	6.97:1 [a] (12.54)	N.D.	11.9	N.D.
$(z^8A, A)_n$	3.0:1	6.48:1 [a] (13.37)	10,736	N.D.	N.D.
$(z^8A, A)_n$	2.0:1	5.03:1 [a] (16.6)	N.D.	N.D.	N.D.
$(z^8A, A)_n$	2.0:1	4.99:1 [a] (16.7)	11,580	9.4	48.9
$(z^8A, A)_n$	1.9:1	4.7:1 [b] (17.6)	11,393	N.D.	42.6
$(z^8A, A)_n$	1.5:1	4.04:1 [a] (19.9)	12,680	7.6	N.D.
$(z^8A, A)_n$	1:1	3.04 [b] (24.75)	N.D.	N.D.	36.9
$(z^8A, A)_n$	1:1	2.87 [b] (25.9)	N.D.	N.D.	36.2
$(z^8A, A)_n$	0.3:1	2.058:1 [b] (32.7)	N.D.	N.D.	N.D.
$(z^8I, I)_n$	2:1	6.905:1 [b] (12.6)	9068	N.D.	variable
$(z^8I, I)_n$	6.17:1	39:1 [a] (2.5)	9479	7.2	variable

(a) Ratio determined via enzymatic hydrolysis as described in 2.2.14a

(b) Ratio determined via alkaline hydrolysis and curve-fitting as described in 2.2.14b

(c) The hyperchromicity of A_n is 50%

Figures in parentheses denote the percentage of z^8N residues in the copolymer

N.D. = not determined

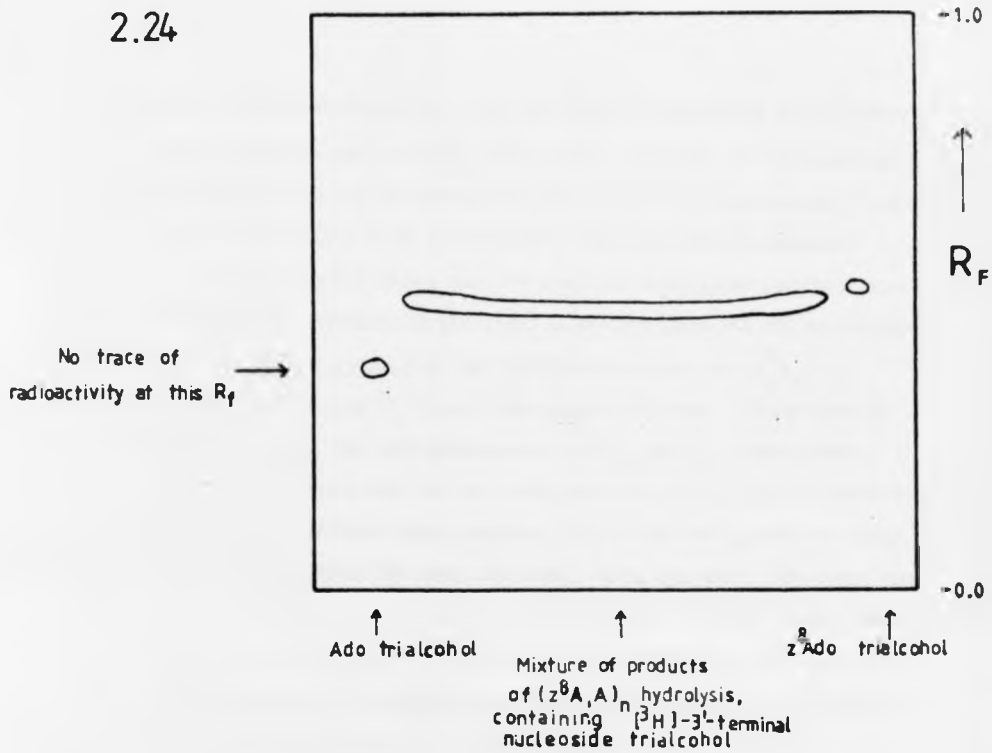
Figure 2.24

Fluorographic detection of 3'-terminal nucleotide in $(z^8A, A)_n$. The [^{3}H]-products were applied as a streak to the tlc plate along with the marker nucleoside trialcohols (for conditions see Section 2.2.15)

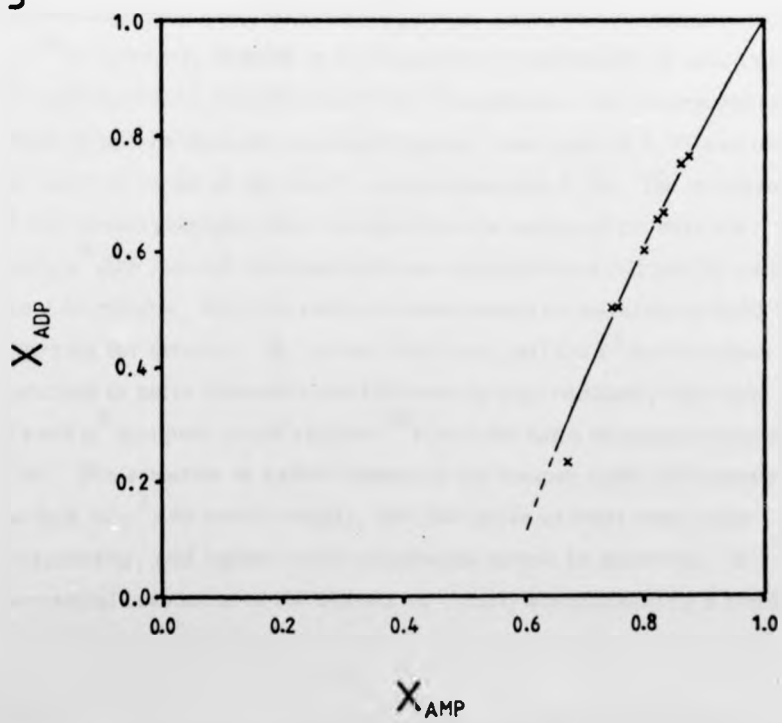
Figure 2.25

A comparison of input/output ratios of nucleotides in a series of $(z^8A, A)_n$ samples. The base composition of polymers was determined as described in Section 2.2.14. X_{ADP} denotes mole fraction of ADP in the polymerisation mixture; X_{AMP} denotes mole fraction AMP residues in the copolymer

2.24


1.0
R_F
0.0
No trace of radioactivity at this R_f
Ado trialcohol
Mixture of products of $(z^8A,A)_n$ hydrolysis, containing $[^3H]$-3'-terminal nucleoside trialcohol
z^8Ado trialcohol

2.25


1.0
0.8
0.6
0.4
0.2
0.0
X_{ADP}
0.0
0.2
0.4
0.6
0.8
1.0
X_{AMP}

chain elongation impaired, but that chain termination always occurs after incorporation of a syn nucleotide. Whether the termination is precipitated by the incorporation of two or more successive 8-azido residues remains to be determined, but would be of interest.

Table 2.4 also shows that the ratio of input base concentration is strikingly different to the ratio in the synthesised polynucleotide. Figure 2.25 is a graph of this input:output data for $(z^8A, A)_n$. Points at (0, 0) and (1, 1) are necessarily defined by the type of plot used, i.e. the homopolymers $(z^8A)_n$ and A_n respectively. The experimental data fall on a slight curve which has an approximate slope of 2. Whether the complete plot would be sigmoid in shape is not evident from the data obtained; it is apparent, however, that the enzyme displays a marked preference for ADP incorporation and that as the mole fraction of ADP in the polymerisation mixture tends to zero, the resulting copolymer tends toward an alternating Az^8A type (i.e. $X_{AMP} \rightarrow \sim 0.5$).

The data in Table 2.5 show the results of a nearest neighbour analysis experiment for $(z^8A, A)_n$. The alkaline hydrolysis of a copolymer synthesised from nucleoside diphosphates, only one of which possesses α-[^{32}P]-linkages, results in a mixture of 3'-nucleotides in which each ^{32}P will have been transferred to its 5' neighbour. In the present case, where it will be seen that an experimental base ratio of 5.03 was obtained, the ratio of counts in the two 3'-nucleotides was 5.75. The interpretation of this result provides some insight into the nature of the reaction. If every z^8Ado residue incorporated was preceded and followed by at least one Ado residue, then the ratio of counts would necessarily be 4.03 (see Appendix for theory). If, on the other hand, all the z^8Ado residues occurred in pairs preceded and followed by Ado residues, then only one of each z^8Ado pair would receive ^{32}P and the ratio of counts would be 9.06. The situation is rather closer to the former case and implies that the bulk of z^8Ado occurs singly, but that pairs at least must exist occasionally, and higher order structures cannot be ruled out. A theoretical treatment of the results is clearly complicated by a number of

Table 2.5

Nearest neighbour analysis of $(z^8A, A)_n$

Purine base	Molar rates of bases in copolymer	Ratio of [^{32}P] in 3'-nucleotides in hydrolysate
z^8A	1	1
A	5.04	5.75

The α-[^{32}P]-containing copolymer was prepared using standard procedure; incubations contained z^8ADP (5 mM), α-[^{32}P]-ADP (10 mM), $MnCl_2$ (4 mM), tris-HCl, pH 9.0 (50 mM) and *E. coli* polynucleotide phosphorylase (3 U), and were held at 45° for 20 hours. The polymer was hydrolysed overnight in 0.3 M sodium hydroxide at 37°, the solution neutralised with HCl and applied to a preparative tlc plate (Merck 60 F_{254}) with upward development in solvent A until the solvent front was near the end of the plate. After drying, the spots were visualised under UV and scraped off to be counted on a suspension in ethoxylated scintillant.

factors. Thus, the input base ratio does not parallel the polymer base ratio and as the polymerisation proceeds the input ratio changes since one of the components is being preferentially utilised. At longer reaction times, the amount of z^8ADP is relatively much higher than at the initiation of synthesis. The enzyme displays a very low preference for the 8-azido nucleotides, and it would appear that treating the occurrence of dimers (and longer sequences) of z^8Ado on a random statistical basis might not be valid because this will not take into account specificity considerations, e.g. after incorporation of a z^8Ado, the next nucleotide to be added will not necessarily be chosen from the pool of NDP's at random. The z^8Ado already in the active site may force the enzyme into adopting a rather different specificity for acceptance of the next nucleotide; such effects are clearly of importance and, in fact, in the PNPase catalysed copolymerisation of 2'-OMe nucleotides in the presence of unsubstituted nucleotides, Rottman and Johnson (1969) found that dimers of the 2'-OMe nucleotide occurred with greater frequency than expected on the grounds of random incorporation.

If one makes the assumption that nucleotides are incorporated into the polymer in a random fashion, with a probability of incorporation directly proportional to the base ratio found by experiment, then the nearest neighbour analysis would theoretically have been 4.51 (see Appendix). That the observed value is higher than this shows he arguments concerning specificity of incorporation and changing concentrations of nucleotides, advanced above, to have some validity. In particular, it is obvious that dimers and higher order sequences of z^8Ado occur with a larger than expected frequency. In connection with this point, an analysis of average chain length by end to total phosphorus determination gave a result of 435 residues average; this is too long to account for the observed nearest neighbour results as a consequence of dimeric or trimeric occurrence of z^8Ado at the 3'-terminus. Calculation shows (see Appendix) that of the ca. 72 z^8Ado residues in such a polymer, approximately 66 would be followed by Ado in the 3'-position, assuming random incorporation. The nearest neighbour results, however, show that in practice only ca. 54 z^8Ado residues are so positioned, and that up to 18 z^8Ado must occur in dimeric or higher order structures. If the analysis is made from the

standpoint of the nearest neighbour "affecting the probability of incorporation of the real nucleotide", it is found that the percentage of z^8Ado that occurs in dimers or higher aggregates is significantly larger, but that the theoretical value for the number of z^8Ado not bounded by 3'-Ado is still some residues too high (see Appendix). It is thus clear that the enzyme is able to maintain polymerisation even after the consecutive additions of z^8Ado. Chain termination must therefore follow either higher order incorporation of z^8Ado (since it has been shown that z^8ADP cannot be homopolymerised), or rest upon some other combination of effects. It is concluded, however, from the 3'-terminal analysis that one of the major determinants of chain determination is the incorporation of the atypical 8-azido nucleotide.

A number of experiments was performed in order to gain information about the solution structure of the copolymers compared to the unsubstituted polymers. The hyperchromism of the $(z^8A, A)_n$ polymer, as determined by alkaline hydrolysis, was comparatively large (see Table 2.4) in analogy with the 50% for poly A (Fresco and Klemperer, 1959). The dependence of the hyperchromism on the z^8Ado content of the polymer would be in accord with a model in which the base-stacking interactions are somewhat impaired in the region of the z^8Ado residues, presumably because in the random coil these bases are likely to exist in an equilibrium of *syn* and *anti* forms with the *syn* predominating.

Thermal transition profiles of the polymers (Figure 2.26) at neutral pH support this view. Thus A_n and $(z^8A, A)_n$ exist in random coils (non-co-operative transitions), whilst the situation for I_n and $(z^8I, I)_n$ is rather more complex. At high salt concentrations, I_n exists as a four-stranded helix, but at lower ionic strength a partially structured form occurs (Thiele and Guschlbauer, 1973). At 0.16 M sodium ion concentration the thermal transition for I_n shows a low but finite degree of co-operativity indicating partial structure, whilst virtually no hyperchromism is seen for $(z^8I, I)_n$. This again implies that alterations in structure have been induced by the azido nucleotide, probably by the occurrence of a *syn* conformation.

When the pH of the solution is lowered sufficiently, the spectrum of A_n undergoes a marked hypochromism and a double stranded parallel protonated helix is formed (Fresco and Klemperer, 1959;

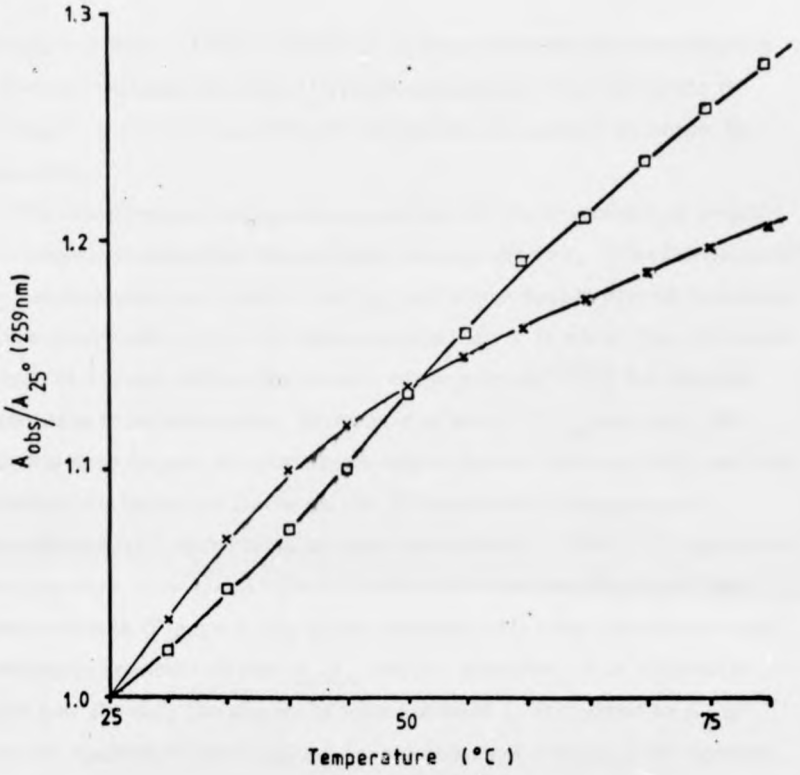
Figure 2.26

Thermal transitions of single stranded polynucleotides

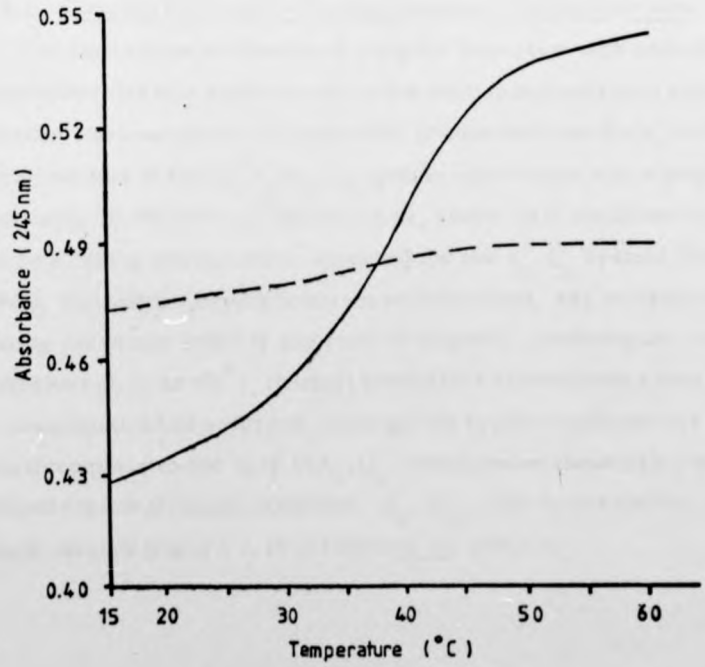
(a) $(z^8A, A)_n$ (X – X – X) and A_n (□ – □ – □) in 0.11 M Na^+, pH 7.0

(b) $(z^8I, I)_n$ (-------) and I_n (———) in 0.16 M Na^+, pH 7.0

a)


1.3
1.2
1.1
1.0
$A_{obs}/A_{25°}$ (259nm)
25
50
75
Temperature (°C)

b)


0.55
0.52
0.49
0.46
0.43
0.40
Absorbance (245nm)
15
20
30
40
50
60
Temperature (°C)

Steiner and Beers, 1959). Figure 2.27 demonstrates that introduction of 8-azido residues into the A_n strand destabilises this structure to lower pH, and in the case of high content of z^8A appears to inhibit its formation.

The experiments lead to the conclusion that incorporation of 8-azido nucleotides destabilises the polymer to some degree. That the expected syn conformation can alter to the anti and allow double strand formation can be inferred from the acid titration data, but it is clear that threshold values of 8-azido nucleotide content of the polymer exist for normal structures to be attainable. In the case of the $(z^8I, I)_n$ polymer, the sensitivity to degree of substitution appears to be very marked, and this conclusion is borne out by the results from complex formation with complementary homopolynucleotides (see below). The C.D. spectra of the polymers show quantitative differences in the magnitudes of their Cotton effects (Figure 2.28) but are qualitatively very similar to those previously reported (Wolfe et al., 1969). However, it is noticeable again how the very low degree of substitution of I_n compared to A_n by 8-azido nucleotide leads to comparable orders of change in the spectra. The generally lower magnitude of the Cotton effects certainly implies a more random form with lowered stacking interactions.

2.3.6 Complex formation with complementary polynucleotides

The quantitative evaluation of complex formation with complementary polynucleotides was made by use of the continuous variation method at a constant total nucleotide concentration (Felsenfeld and Rich, 1957). The initial studies on the $(z^8A, A)_n.U_n$ system were made with a polymer containing 25.9% of the z^8Ado residues. Under salt conditions (0.06 M Na^+), where a strong interaction is observed for the $A_n.U_n$ system (Blake et al., 1967), virtually no hyperchromism was observed, and no break in the mixing curve was noted at any ratio of polymer. Under higher salt conditions (0.11 M Na^+), thermal transitions showed that a small degree of complexation had occurred, although the hyperchromism was very small compared to the case of $A_n.U_n$ which, under these salt conditions, formed a triple stranded structure, $A_n.2U_n$, with direct melting to the single strands (Figure 2.29 and Blake et al. (1967)).

Figure 2.27

pH titration of adenosine-containing polynucleotides.

A_n (X − X − X); $(z^8A, A)_n$ containing 13.4% z^8Ado
(O − O − O); $(z^8A, A)_n$ containing 19.9% z^8Adb
(□−□−□)

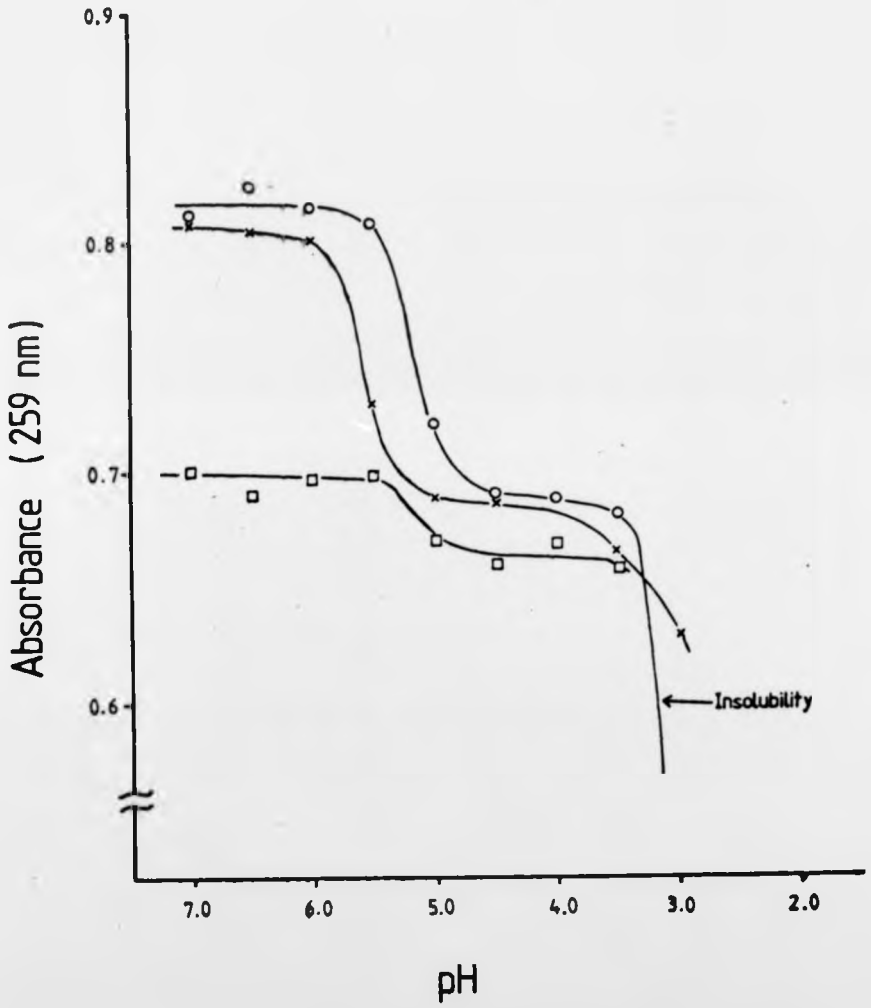

0.9
0.8
0.7
0.6
Absorbance (259 nm)
Insolubility
7.0
6.0
5.0
4.0
3.0
2.0
pH

Figure 2.28

Circular dichroism spectra of synthetic polynucleotides

(a) A_n (———) and $(z^8A, A)_n$ containing 12.5 % z^8Ado (------)

(b) I_n (———) and $(z^8I, I)_n$ containing 2.5% z^8Ino (------)

Spectra were recorded at 20° in phosphate buffered saline

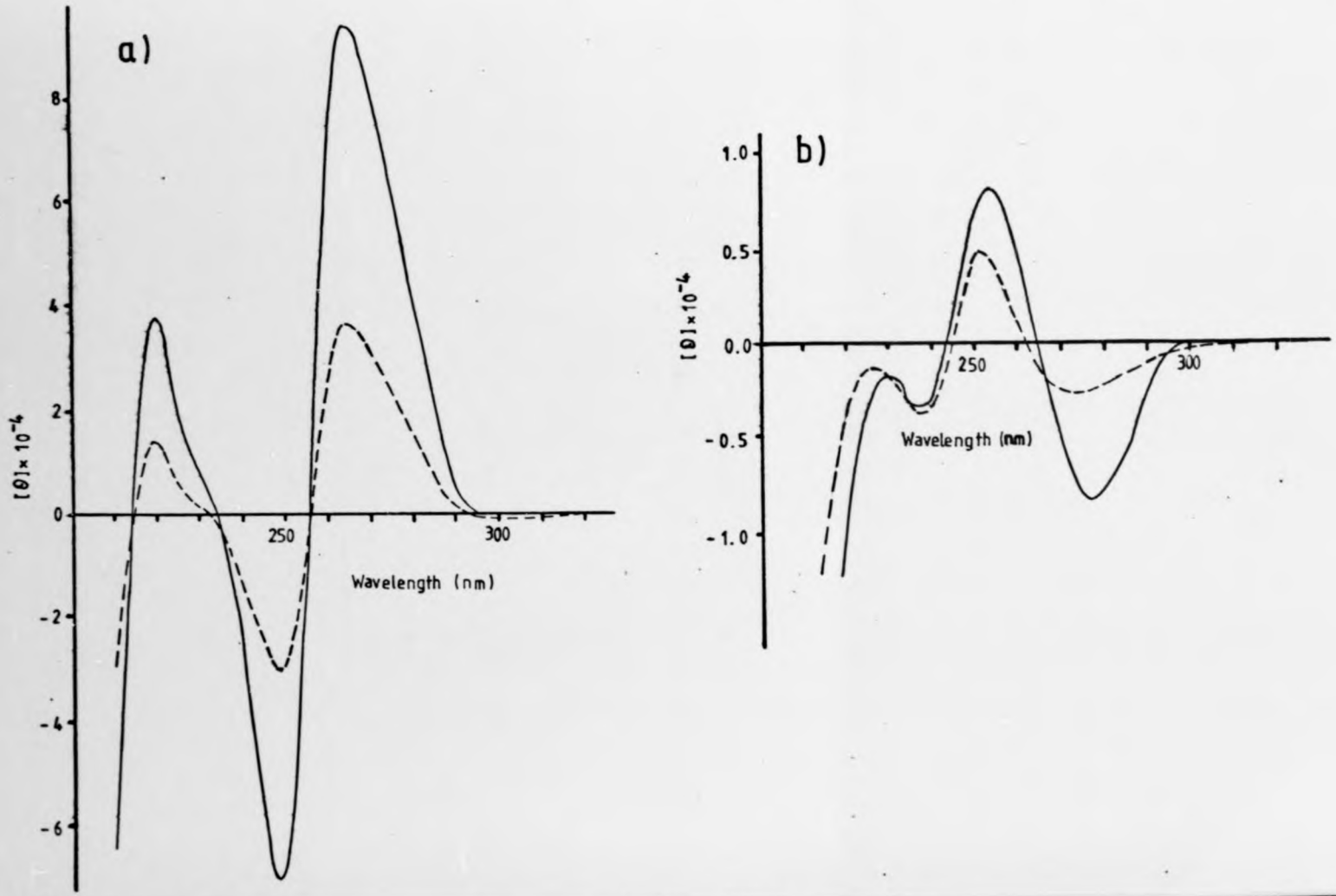
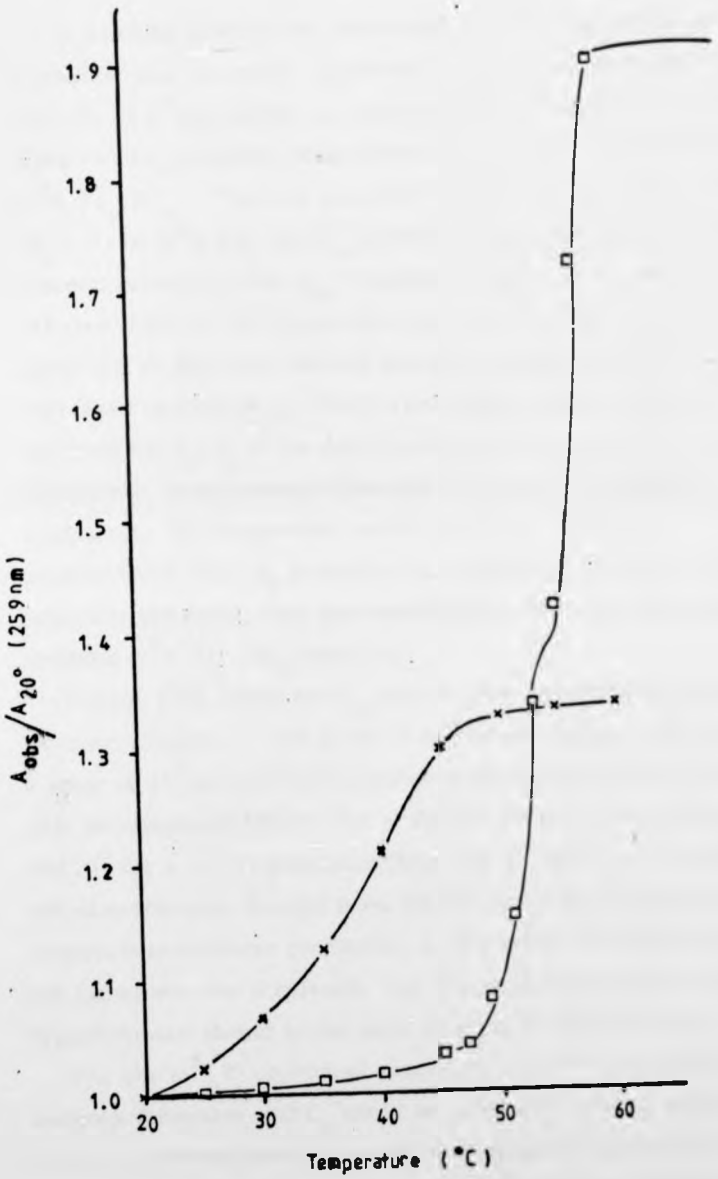

a)
8
6
4
2
0
-2
-4
-6
[θ]×10⁻⁴
250
300
Wavelength (nm)
b)
1.0
0.5
0.0
-0.5
-1.0
[θ]×10⁻⁴
250
300
Wavelength (nm)

Figure 2.29

Thermal transition of a 1:1 mixture of U_n and $(z^8A.A)_n$ (containing 25.9% z^8Ado) recorded in 0.01 M sodium cacodylate, 0.1 M sodium chloride, pH 7.0 (X – X – X). The data for an $A_n.U_n$ mixture (□ – □ – □) at 0.1 M Na^+ are adapted from Blake et al. (1967)


1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
$A_{obs}/A_{20°}$ (259 nm)
20
30
40
50
60
Temperature (°C)

In samples in which the percentage of z^8Ado was lower, good complex formation was, however, observed. Figure 2.30 shows that for a sample with 16.7% z^8Ado content, complex formation occurs at X_n 0.67; in other words, the stable form under these conditions is the triple strand $(z^8A,A)_n.2U_n$. Thermal transition profiles of equimolar mixtures (X_n 0.5) of $(z^8A,A)_n$ and U_n under conditions of increasing Na^+ concentration show that T_m increases (Figure 2.31), but that at low salt concentration the hypochromism of the complex is smaller (this latter will be discussed further below). Comparison of the T_m profiles with those of Blake et al. (1967) show that the duplex form, so readily observed for $A_n.U_n$ at low salt concentration by two-step thermal transitions, is completely absent for $(z^8A,A)_n.U_n$ under these conditions. It is noteworthy in this respect that the T_m of a 0.5 X mixture and a 0.67 X_n mixture at 0.1 M sodium ion concentration are precisely the same, both presumably being composed of a triple-stranded $(z^8A,A)_n.2U_n$ complex.

Figure 2.32 shows the T_m data plotted versus the logarithm of sodium ion concentration. The points at higher salt fall on a straight line with a slope of 24° per ten-fold increase in sodium ion concentration. This is to be compared with a value of 26° for the (3 → 1) transition of An.Un and 20° for a (2 → 1) transition (Blake et al., 1967). The points at low salt concentration diverge from the line by virtue of incomplete complex formation under these conditions, an inference strengthened by the very low hypochromism displayed. For full complex formation, the total hypochromism should be the same at all salt concentrations.

For the (z^8I,I) copolymer containing 12.6% z^8Ino residues, no complex formation with C_n could be observed, even by monitoring the spectra of the mixtures at various wavelengths (Figure 2.33). In a co-polymer in which the percentage of z^8Ino was rather lower (2.5%), there was, however, good duplex formation as monitored at different wavelengths and at different salt concentrations (Figure 2.34). The dependence of T_m on sodium ion concentration was investigated, and showed rather similar behaviour to that for the $(z^8A,A)_n.U_n$ complex

Figure 2.30

Jobplot of mixtures of $(z^8A, A)_n$ (containing 16.7% z^8Ado) and U_n in 0.01 M sodium cacodylate, 0.1 M sodium chloride, pH 7.0

245 nm (□ – □ – □) and 259 nm (X – X – X)

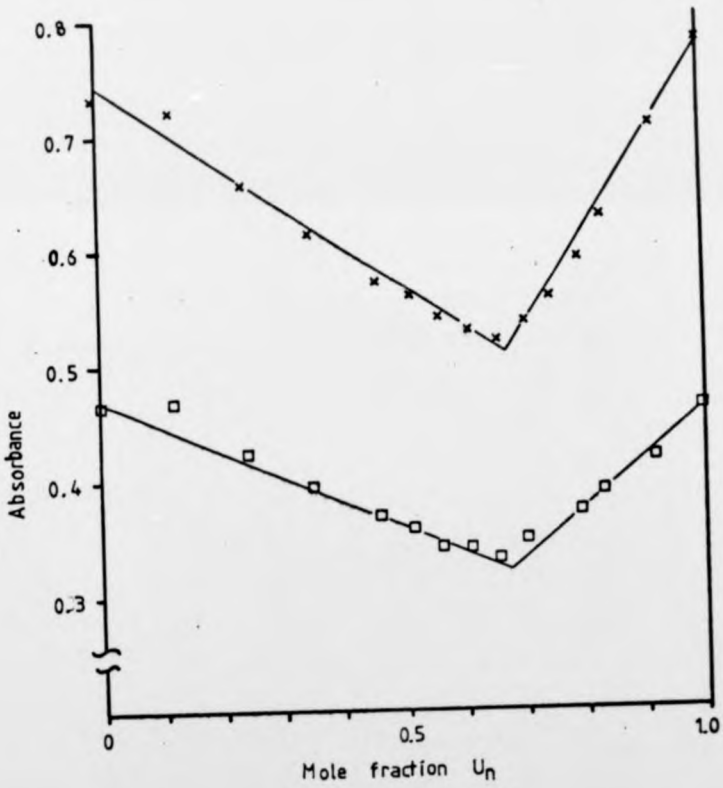

0.8
0.7
0.6
0.5
0.4
0.3
Absorbance
0
0.5
1.0
Mole fraction U_n

Figure 2.31

Thermal transitions of 1:1 mixtures of U_n and $(z^8A, A)_n$ (containing 16.7% z^8Ado) at a series of Na^+ concentrations

(a) 0.05 M

(b) 0.10 M

(c) 0.20 M

(d) 0.46 M sodium phosphate, pH 7.0

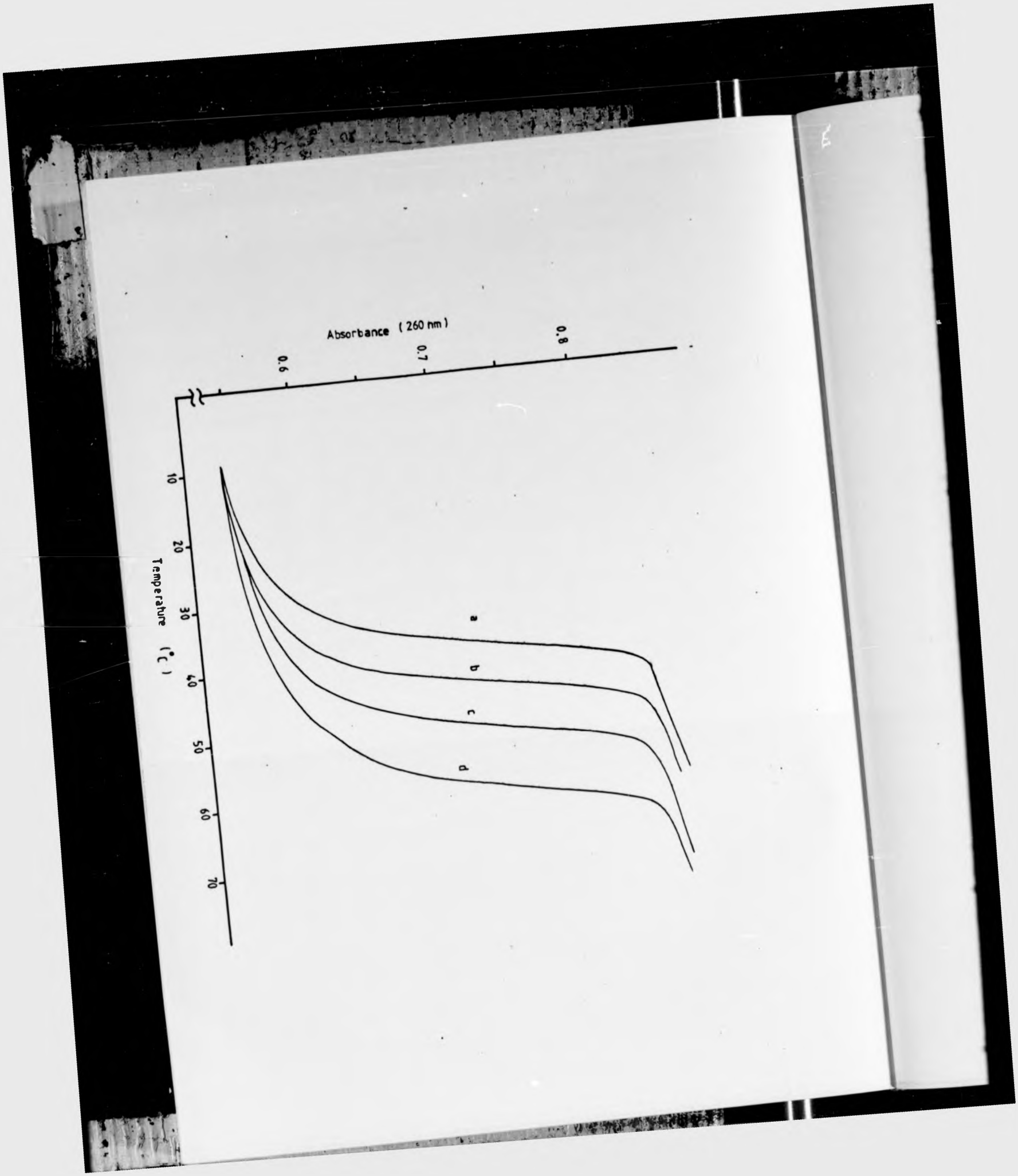

Absorbance (260 nm)
0.6
0.7
0.8
10
20
30
40
50
60
70
Temperature (°C)
a
b
c
d

Figure 2.32

The variation of T_m with sodium ion concentration for 1:1 mixture of $(z^8A, A)_n$ and U_n as described in Figure 2.31

Figure 2.32

The variation of T_m with sodium ion concentration for 1:1 mixture of $(z^8A, A)_n$ and U_n as described in Figure 2.31

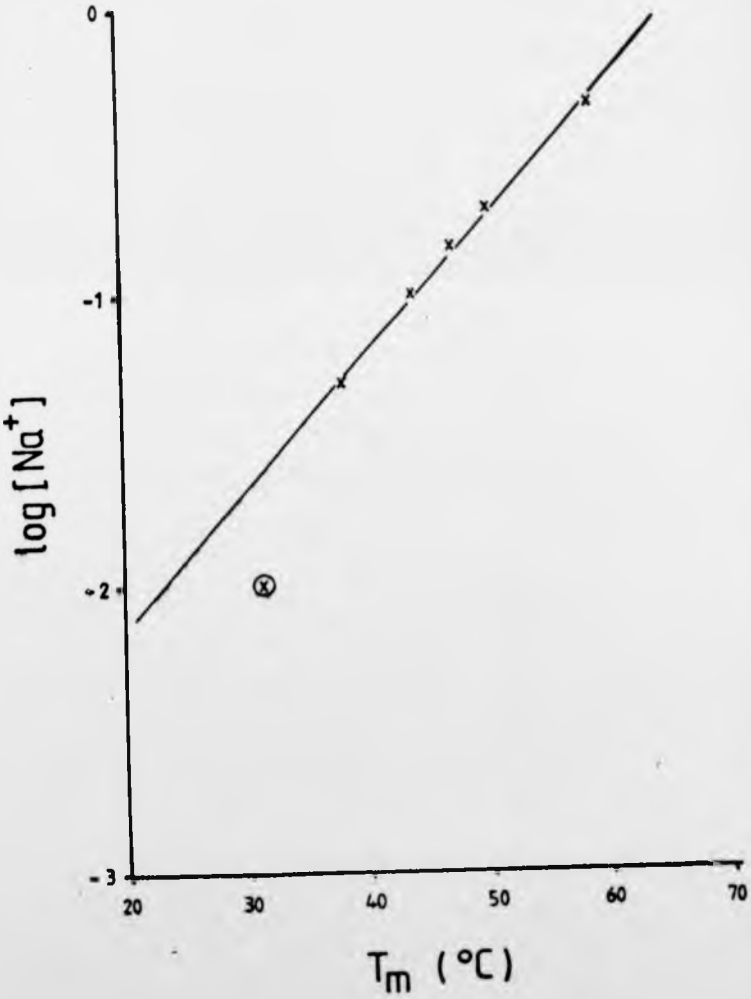

0
-1
-2
-3
log [Na+]
20
30
40
50
60
70
Tm (°C)

Figure 2.33

Job plot for mixtures of $(z^8I, I)_n$ (containing 12.6% z^8Ino) and C_n in 0.01 M sodium cacodylate, 0.1 M sodium chloride, pH 7.0

240 nm (+ −+ − +) and 280 nm (X − X − X)

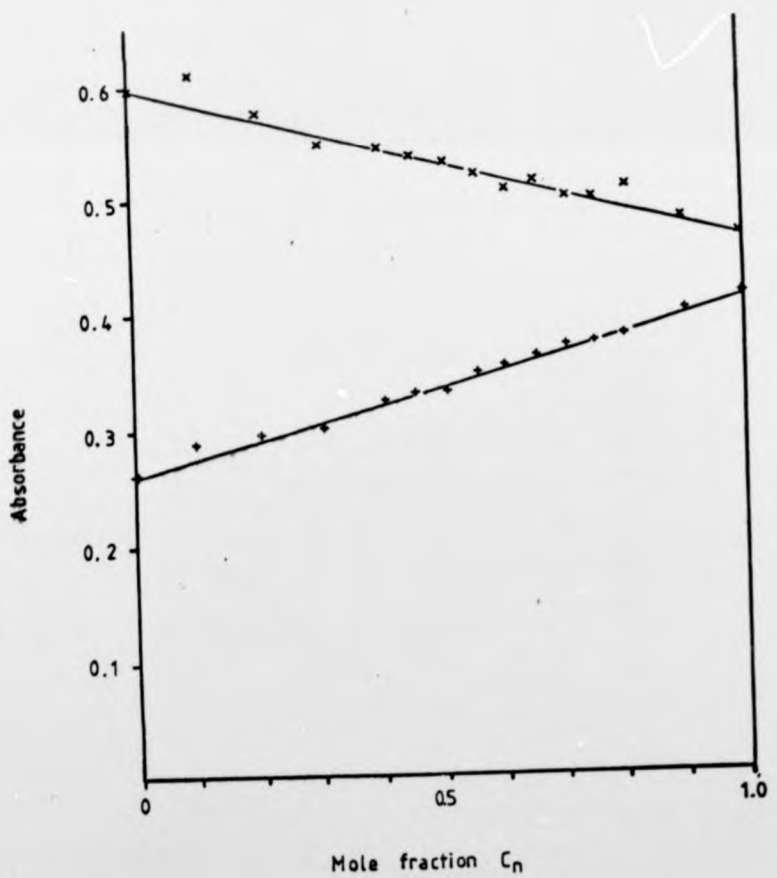

0.6
0.5
0.4
0.3
0.2
0.1
Absorbance
0
0.5
1.0
Mole fraction C_n

Figure 2.34

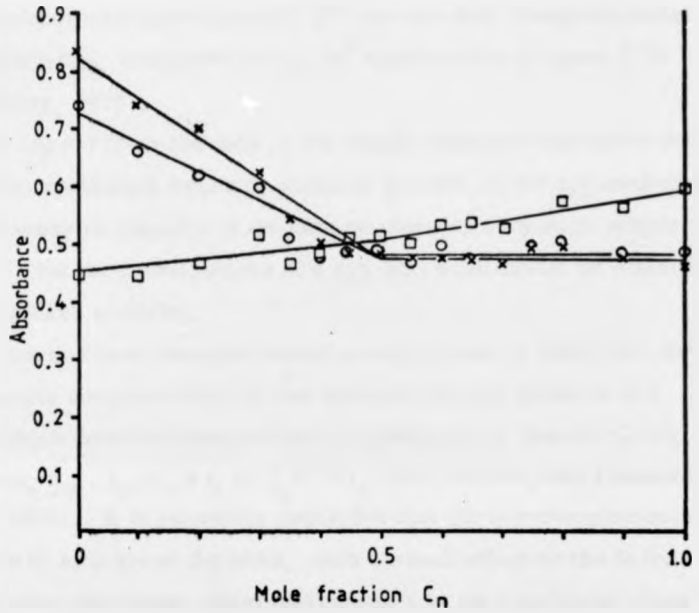
Jobplot for mixtures of $(z^8I, I)_n$ (containing 2.5% z^8Ino) and C_n in

(a) 0.05 M sodium phosphate, pH 7.0

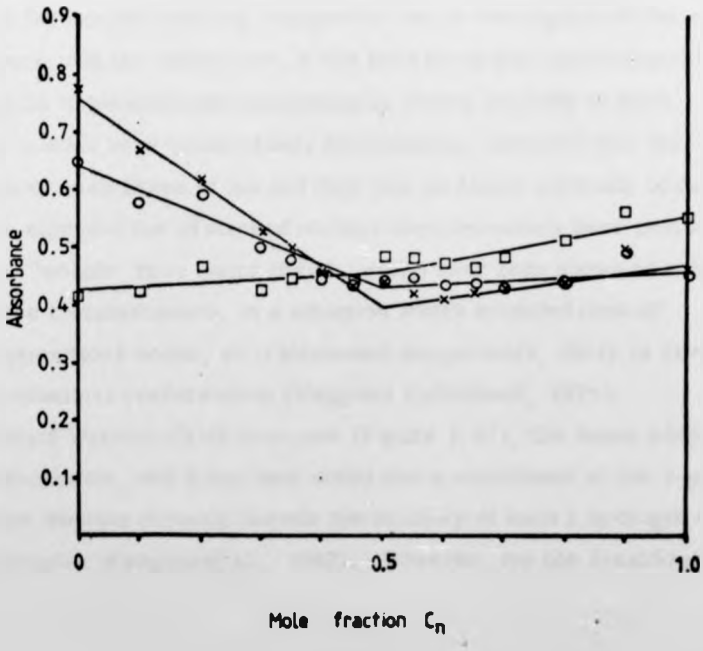
(b) 0.25 M sodium phosphate, pH 7.0

245 nm (X – X – X), 255 nm (O – O – O) and 265 nm (□ – □ – □)

a)


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance
0
0.5
1.0
Mole fraction C_n

b)


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance
0
0.5
1.0
Mole fraction C_n

above, i.e. decreased hypochromism at the lower salt concentrations indicating incomplete duplex formation (Figures 2.35 and 2.36). The T_m dependence was approximately 17^o per ten-fold change in sodium ion concentration, compared to ca. 20^o expected for a duplex RNA (Guschlbauer, 1976).

It was argued from the data on the single stranded copolymer that the 8-azido nucleoside residues probably existed in the syn conformation. It is necessary to consider if the data on complex formation support this view, and what the consequences of a syn-anti equilibrium on stability of such complexes would be.

Many studies have been performed on complexes in which one strand of a normally complemetary duplex contains various amounts of a residue which does not form normal complementary Watson-Crick base-pairs, e.g. $I_n.(C,A)_n$ or $I_n(C,U)_n$ (for a review, see Lomant and Fresco, 1975). It is generally concluded that the non-complementary residues will loop out of the helix, with a small effect on the helix stability, since previously separated residues in the copolymer strand are now accommodated contiguously in base pairs with the complementary homopolymer strand. The loops are sterically compatible with such a structure and the degree of destabilisation produced corresponds to the amount of favourable stacking interaction lost on assumption of the extra-helical form. On the other hand, it has been found that some atypical bases can be accommodated intrahelically if they are able to form hydrogen-bonded base-pairs of only low stability, provided that the proportion of such bases is low and that this incidence normally occurs only in an extended run of stacked normal complementary base pairs. Thus, the "wobble" base pairs (Crick, 1966) have been shown to exist under these circumstances. In a situation where extended runs of wobble oppositions occur, or at increased temperature, there is reversion to the extrahelical conformation (Wang and Kallenbach, 1971).

In normal Watson-Crick base pair (Figure 2.37), the bases adopt an anti conformation, and it has been noted that a substituent at the 8-position of a purine does not directly impede the stability of such a hydrogen-bonded complex (Kyogotu et al., 1967). However, for the 8-azido purine

Figure 2.35

Thermal transitions of 1:1 mixtures of C_n and $(z^8I, I)_n$ (containing 2.5% z^8Ino) at a series of Na^+ concentrations.

(a) 0.01

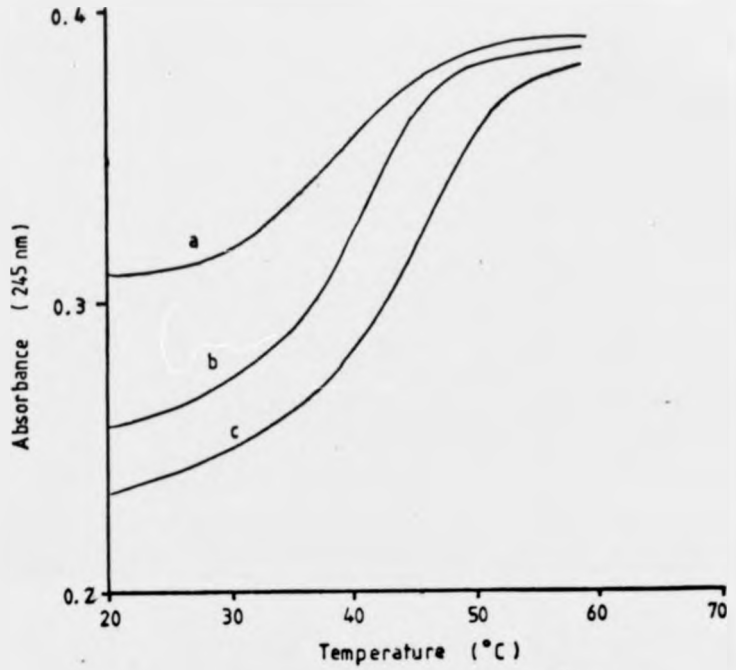
(b) 0.02

(c) 0.05

(d) 0.10

(e) 0.20

(f) 0.50 M sodium phosphate, pH 7.0


0.4
0.3
0.2
Absorbance (245 nm)
a
b
c
20
30
40
50
60
70
Temperature (°C)

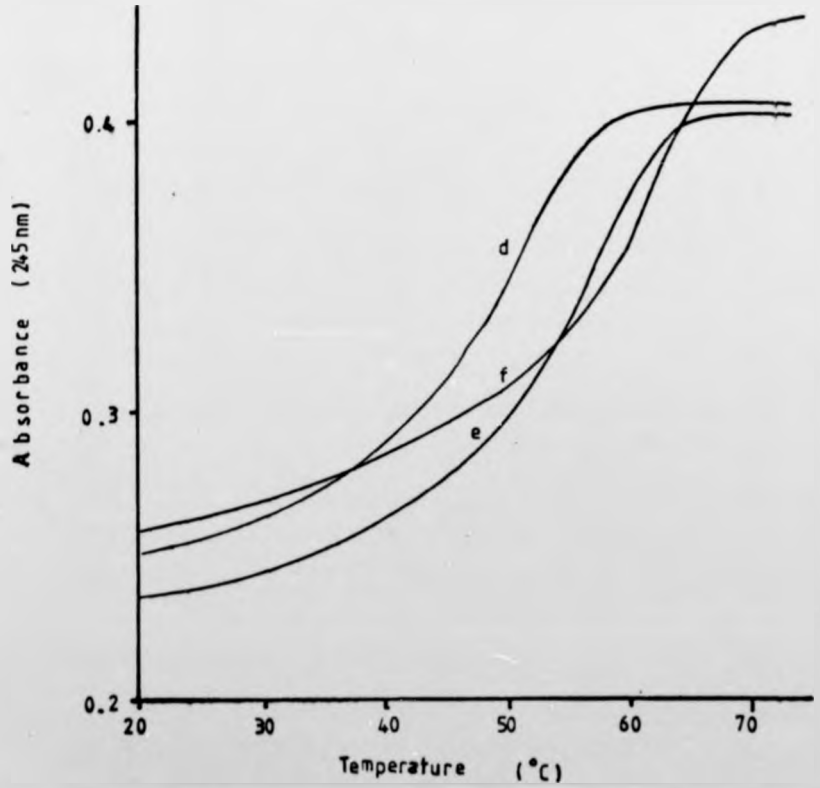
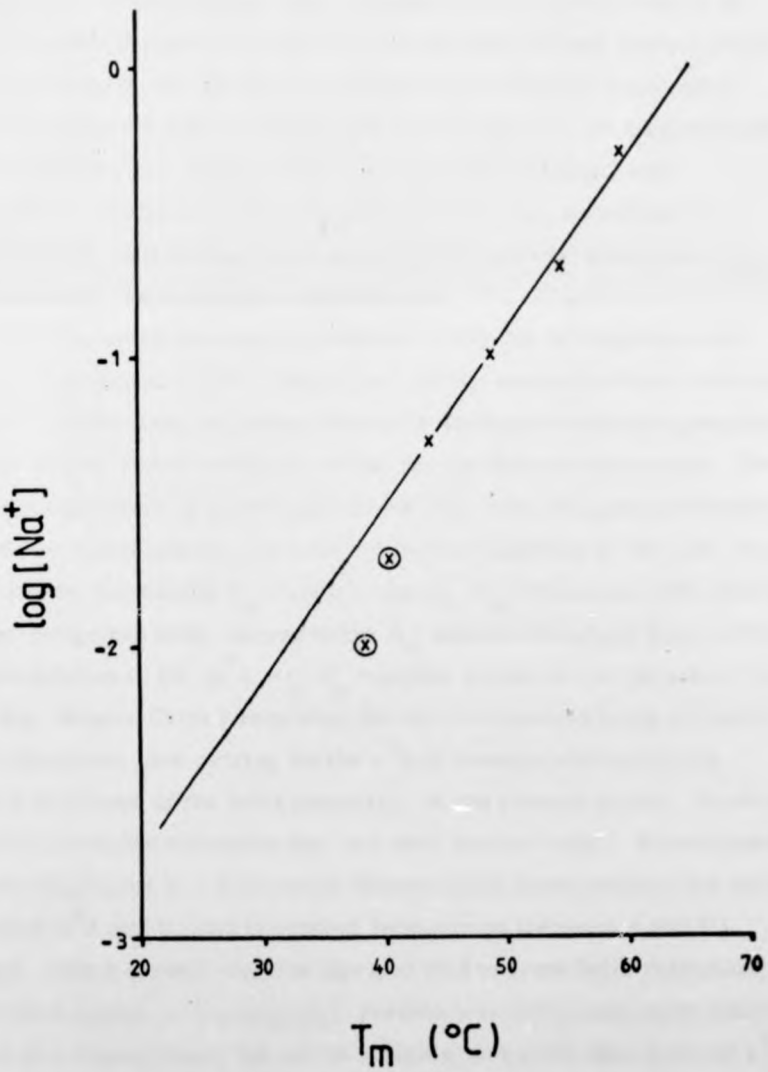

0.4
0.3
0.2
Absorbance (245 nm)
d
f
e
20
30
40
50
60
70
Temperature (°C)

Figure 2.36

The variation of T_m with sodium ion concentration for 1:1 mixtures of $(z^8 I, I)_n$ and C_n as described in Figure 2.35


0
-1
-2
-3
log [Na+]
20
30
40
50
60
70
Tm (°C)

bases to adopt Watson-Crick base pairing, a shift of the syn $\rightleftharpoons$ anti equilibrium completely in favour of the anti would be required. This would obviously lead to a degree of destabilisation that would be offset, below a certain level of 8-azido purine incorporation, by the gain in free energy achieved by the hydrogen-bonding of a fully base paired structure. Each hydrogen bond formed would provide an energy of stabilisation of approximately 5 kcal/mole (Lomant and Fresco, 1975). As noted above, the barrier to rotation in adenosine is of the order of 6-7 kcal/mole: this is presumably a little higher in an 8-substituted purine nucleotide. Thus, the two energies are of comparable magnitude, and which structure predominates, i.e. extrahelical or intrahelical, will depend to a large extent on external conditions, e.g. temperature, ionic strength and solvation.

An alternative base pairing scheme is that due to Hoogsteen and depicted in Figure 2.38. The purine residue exists in what is formally a syn conformation, assuming the ribose-phosphate backbone geometry of the double strand is similar to that for the Watson-Crick case. The possible existence of such a pair in the I.C case requires protonation, but there appears to be no reason for its non-existence in the A.U case, and indeed, the second U_n strand in the $A_n.2U_n$ triplex has been shown to be Hoogsteen base paired to the A_n strand (Arnott and Bond, 1973). Consideration of the $(z^8A,A)_n.U_n$ complex shows that in the initial 1:1 duplex, Watson-Crick base pairing for the A.U contacts might co-exist with Hoogsteen base pairing for the z^8A.U contacts with relatively small distortion of the helix geometry. In the present studies, however, such 1:1 complex formation has not been found to exist. In the triplex, there would again be a mixture of Watson-Crick base-pairing (this time between z^8A and U) and Hoogsteen base pairing (between A and U). Again, such a system would be likely to lead to some helix distortion, and there appear to be no a priori reasons why such a structure would exist in a triplex form, but not in a duplex, at a given base ratio of z^8A to A. Since the I.C system at neutral pH cannot form Hoogsteen base-

Figure 2.37

Watson-Crick base pairs for A.U and I.C

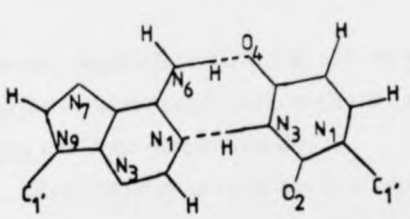
Figure 2.38

Hoogsteen base pairs for A.U and I.C. (requires protonation)

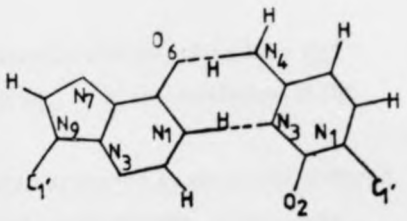
Figure 2.39

A triple stranded structure for z^{8}A.2U with the combination of Watson-Crick and Hoogsteen base pairing

2.37

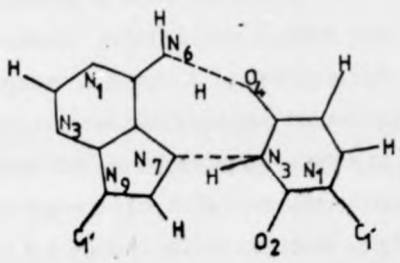


A – U

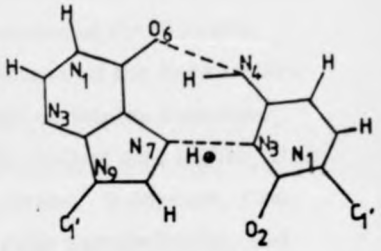


I – C

2.38

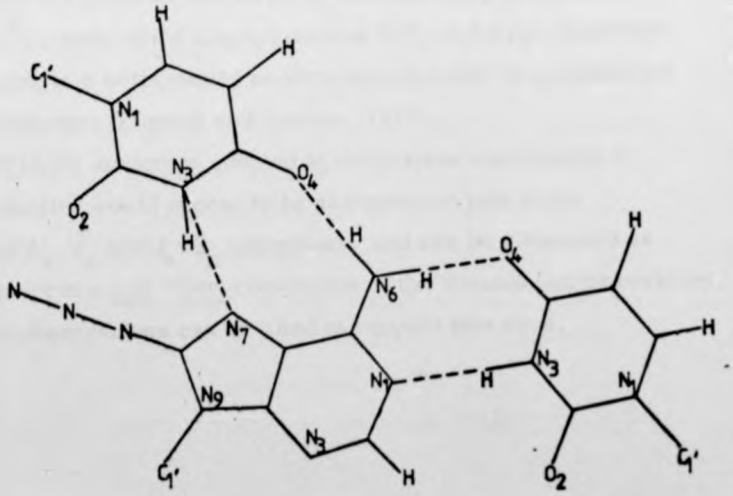


A – U



I – C

2.39



pairs, duplex formation can only be visualised in this case by a syn → anti conversion and Watson-Crick pairing, or accommodation of the syn residues extrahelically.

Extrahelicity of residues in a copolymer strand is normally deduced from the Job plots of complex formation. Intersection of the lines occurs at points other than 0.5 X for duplex formation and similarly at points other than 0.67 X for triplex formation. In addition, the T_m dependence on sodium ion concentration increases due to the local clustering of shielding cations in the neighbourhood of the extrahelical residues. In both cases studied here, inspection of the Job plots (Figures 2.30 and 2.34) reveals that intersection of the lines occurs very close to that expected for normal duplex or triplex formation, whilst the Tm dependence is seen to be less, rather than higher, than that expected for fully complementary complexes. It appears, then, that the 8-azido purine residues might not exist extrahelically, and this view is supported by further observations reported below.

Ikehara et al. (1969) have studied the complex formation between U_n and a copolymer composed of A and br^8G residues $(A, br^8G)_n$. Job plots show that under conditions of 0.11 M sodium ion concentration, the br^8G residues must loop out in an extrahelical fashion. Addition of 0.01 M magnesium ion allows an intrahelical state to be achieved. In this case, however, not only does the br^8G exist normally in a syn conformation, but the base pair formed via syn → anti conversion is "wobble" type and hence of intrinsically low stability, particularly since the br^8G content of the copolymer was 23%, and large quantities of wobble pairs in a helix should be very unfavourable to maintenance of a stable structure (Lomant and Fresco, 1975).

The most likely structure adopted by the 8-azido copolymers in complex formation would appear to be analogous to that of the unsubstituted $A_n.U_n$ and $I_n.C_n$ complexes, and can be visualised as the consequence of a syn → anti conversion of the 8-azido purine residues. A number of observations can be cited to support this view.

The apparent low co-operativity of the T_m profiles at the beginning of the hyperchromic change is not altered at higher salt concentration, nor are the transition breadths affected by this change. Both phenomena are related to base pair compositional heterogeneity (Lomant and Fresco, 1975), but not extrahelicity. In an extrahelical complex the co-operativity of dissociation is markedly affected by sodium ion concentration, and the transition breadth is also responsive to such increments, becoming much lower at high sodium ion concentration. Thus, in the present investigation, the early part of the T_m profile probably represents melting of $z^8A.U$ and $z^8I.C$ base pairs; these would obviously melt at a lower temperature due to the destabilising effect of the syn $\rightleftharpoons$ anti equilibrium asserting itself.

The poor complex formation observed at low salt concentration reflects the inadequate free energy gain obtained by Watson-Crick base pairing (an electrostatic phenomenon and hence sensitive to ionic conditions) compared to the energy required to maintain the 8-azido bases in the anti conformation under these conditions. If the 8-azido residues were extrahelical, then we should expect to see full complex formation, albeit stable at correspondingly lower temperatures. This was not observed.

The inability of the $(z^8A, A)_n$ copolymer to form 1:1 complexes with U_n deserves mention. It is assumed that because of the high proportion of bases of the 8-azido type present in the particular sample studied (16.7%), the free energy gain in a 1:1 complex is not sufficient to maintain the anti conformation even at high salt concentration. The addition of a second U_n strand in a Hoogsteen pairing system (Figure 2.39) provides a large increase in free energy, sufficient to maintain an integral system. This is not to say that the 1:1 complex could not enjoy an existence under suitable conditions of salt and temperature, but such a complex was not detected in the present investigation. A rather low proportion of z^8A in the co-polymer would probably allow 1:1 complex formation to be easily observed. Thus, in the case of $(z^8I, I)_n.C_n$, 12.6% of z^8I produced no complex formation, but 2.5% z^8I gave an easily observable duplex. This latter might also be a

reflection of the rather lower tendency, seen in the co-polymer itself, of z^8Ino adopt conformations suitable for stacking or hydrogen bonding interactions.

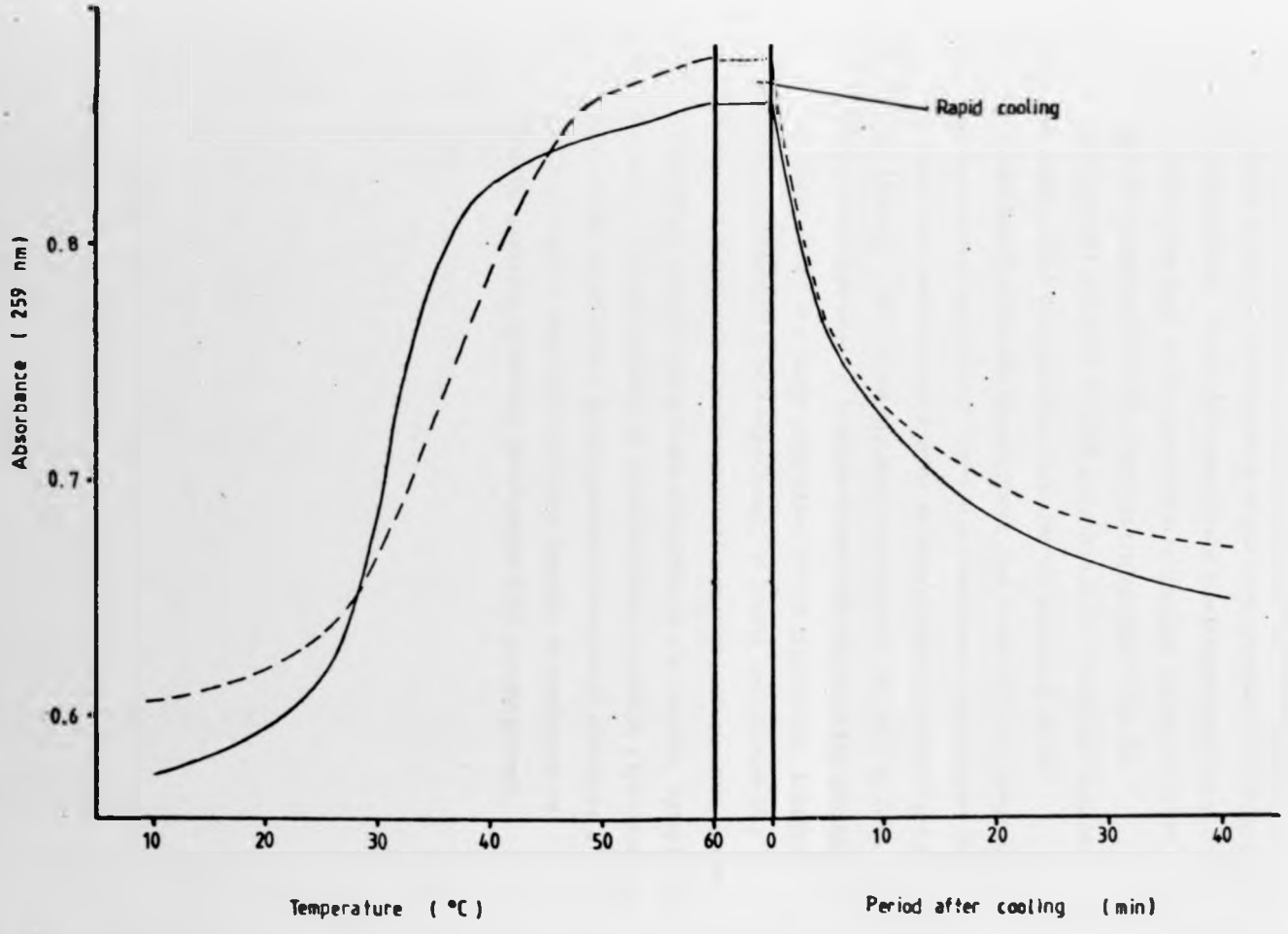
Finally, the existence of 8-azido purine residues in a Hoogsteen base pair involves a rather close opposition of the azido group with the 2-keto group of the uracil base. Irradiation of a 1:1 mixture of $(z^8A, A)_n$ and U_n was performed to see if cross-linking of any of the strands could be achieved by nitrene generation and insertion into contact residues. This potential cross-linking was studied by the renaturation kinetics of a fully denatured complex (Figure 2.40). The experiment was performed at low salt concentration (0.01 M Na^+), since at higher salt, re-annealing is extremely rapid for homopolymer complexes. No elevated rate of renaturation was observed, although it is of some interest to note that the product of irradiation has a higher T_m than the starting material. Chemically cross-linked DNA has been shown to give extremely rapid renaturation (Geiduschek, 1961) under low ionic strength conditions, so that the present result is taken to imply no cross-linking of the complexes. On the other hand, if the Hoogsteen pairing occurred only between two strands and not as a mixture between all three, then cross-linking of two strands would still leave the second U_n strand free, and the slow kinetics of renaturation would still be observed in large part. The higher T_m of the product may be a reflection that the nitrene generated by photolysis has reacted to give a much smaller 8-substituent (e.g. 8-amino by $\dot{H}$-abstraction). Such a grouping would have only small steric requirements, the anti conformation would be much more likely to exist and consequent destabilisation of the complex would be lowered. Thus the absence of observable cross-linking cannot be taken as evidence against the structure of the triple strand as proposed, and the inference to be drawn appears to be that the azido group is constrained exterior to the helix core (see Figure 2.39), and in such a position as likely to be of value in photoaffinity labelling experiments involving interaction between a receptor and the RNA complex.

Figure 2.40

Thermal transition and renaturation kinetics for a 1:1 mixture of $(z^8A, A)_n$ (containing 16.7% z^8Ado) in 0.01 M sodium phosphate, pH 7.0. Rapid cooling of the mixture was achieved from 60^o to 15^o in approximately 3 minutes.

Native hybrid (———)

Hybrid treated with UV to ensure complete destruction of the azido moiety (- - - - - - -)


Absorbance (259 nm)
0.8
0.7
0.6
Rapid cooling
10
20
30
40
50
60
0
10
20
30
40
Temperature (°C)
Period after cooling (min)

The conclusion reached above that the z^8Ado grouping can be constrained to engage in normal Watson-Crick base pairing by adoption of the anti conformation might have important consequences for photoaffinity labelling experiments involving physiological RNA species in the light of the observation by Kapuler and Reich (1971) that certain 8-substituted purine riboside triphosphates can be incorporated into RNA by RNA polymerase in a template-directed reaction. Such incorporation requires the presence of the unsubstituted nucleoside triphosphate, but levels of 2-4% integration were consistently achieved. Since it is a necessary requirement that normal base pairing occurs prior to incorporation, it seems possible that although z^8ATP is not utilised as a substitute for ATP by RNA polymerase (see above), it might enter RNA chains in the presence of excess ATP. In a large population of RNA transcripts, therefore, there would certainly be a significant, if small, percentage of z^8A bases. It is possible that fundamental structural information on the composition of ribonucleoprotein complexes in the nucleus, cytoplasm and at the ribosomes could be obtained from irradiation experiments, since there would exist a small amount of complexes where the z^8A residues were in important sequence specific or conformation specific positions of contact between the RNA and the protein.

CHAPTER THREE

3.1 Introduction

3.1.1 8-Azido nucleotides as photoaffinity labels

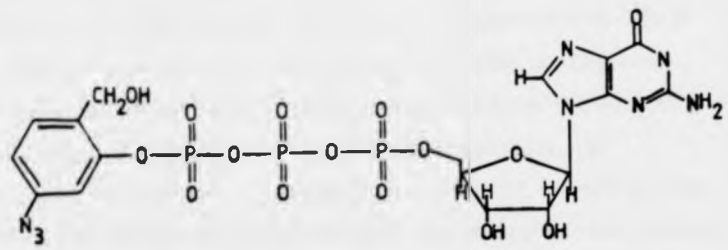
Photoaffinity labelling as a technique was conceived and applied at an early stage in the development of active site directed irreversible inhibition methods (Singh et al., 1962 ; see Chapter One). Since then, recognition of the general application of photoaffinity labelling has created a wealth of research in this area. One area where many investigations have been performed is that concerned with nucleotide requiring systems. Such interest is to be expected in view of the large number of processes that involve nucleotides as an essential element, but which are little understood in terms of their molecular associations, e.g. enzymic systems where nucleotides can act as substrate, allosteric effector or cofactor, cyclic nucleotide receptors in membranes or in intracellular locations, transport mechanisms and involvement in both the nucleic acid transcription/replication and protein synthetic machinery.

A variety of modifications has been made to purine riboside nucleotides to provide photoaffinity labels suitable for these different applications. Attachment via the phosphate moiety has been successfully used to identify a GTP binding protein in E. coli ribosomes by irradiation of P^1-(3-azido-6-hydroxymethylphenyl)-P^3-(5'-guanosyl)triphosphate (I, Figure 3.1) in the presence of EF-G (Maassen and Möller, 1978), while P^1-(4-azidoanilide)-P^3-(5'-adenosyl) triphosphate (II, Figure 3.1) has proved useful in studies of aminoacylation reactions of tRNA synthetase (Ankilova et al., 1975; Akhverdyan et al., 1977). Ribose modification at the 2'-(3')-position by arylazido derivatives has been employed to label mitochondrial ATPase using ADP (Lumardi et al., 1977) and ATP (Russel et al., 1976) substitution. Modification of the base has, however, been largely confined to introduction of the azido group at the 8-position of the purine ring.

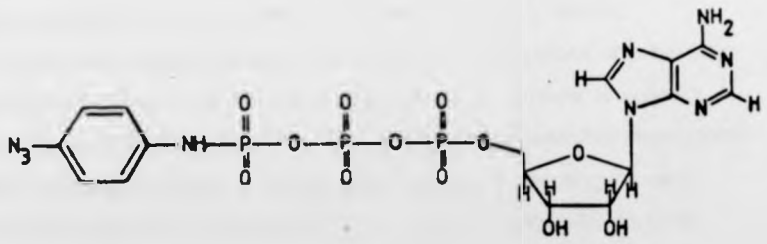
The earliest use of z^8Ado nucleotides in photoaffinity labelling was by Haley and Hoffman (1974) who investigated the interaction of z^8ATP with ATPases of erythrocyte ghosts. Both the (Na^+, K^+)- and the Mg^{2+}-

Figure 3.1

Structure of two nucleotidic, azide-containing photoaffinity labels used successfully for labelling of macromolecules (see text).



I



II

ATPase interacted in a specific and reversible manner with the analogue, and photolysis allowed the subsequent isolation by SDS electrophoresis of selected protein components attached to the affinity label. This labelling could be abolished in competition with the normal substrate. It was felt that such z^8Ado nucleotides were highly effective, fulfilling the criteria required for photoaffinity labels and likely to prove a powerful tool in probing nucleotide binding sites.

This suggestion has received substantial support from the number of successful reports of their use in a variety of systems. In particular, Haley has pursued studies with a range of 8-azido purine nucleotides and has probed the cAMP binding sites on cellular membranes (Haley, 1975; Owens and Haley, 1976), at intracellular locations (Skare et al., 1977) and subunit localisation on protein kinases (Pomerantz et al., 1975), whilst guanosine nucleotide interactions with the proteins of microtubules have been investigated with z^8GTP (Geahlen and Haley, 1976). In other studies the subunit structure and localisation of binding sites for ATP in ATPase complexes has been investigated both for mitochondrial (Wagenvoord et al., 1977) and bacterial (Scheurich et al., 1978) enzymes, while proteins responsible for adenine nucleotide translocation in mitochondrial energy requiring processes have been identified (Schäfer and Penades, 1977; Heaton et al., 1978).

It has become apparent that the detailed investigation of a binding site in terms of location of those amino acids important in contact, binding or catalytic functions is not immediately amenable from photoaffinity labelling studies. Detailed investigations on trypsin and chymotrypsin after photolysis of the diazoacyl intermediates have revealed labelling of a cysteine residue in both cases (Hexter and Westheimer, 1971). However, Bridges and Knowles (1974), investigating the same system found on analysis a number of radioactive peptides arising from photolysis of 4-azido [^{14}C] cinnamoyl α-chymotrypsin and several peptides were found in a similar study on subtilisin (Stefanovsky and Westheimer, 1973). The photochemistry of insertion of carbenes or nitrenes into amino acid residues is very little

understood, so that identification of altered amino acids presents difficulties and is likely to be attempted by difference in amino acid analysis rather than by positive identification. Hixson and Hixson (1975) reported their inability to obtain specific labelling patterns in a variety of enzyme-substrate systems, and came to the conclusion that such studies were only likely to provide ambiguous information.

The success of photoaffinity labelling has stemmed from its ability to detect binding interaction in complex systems where a detailed characterisation of the binding site is not required. In many cases identification of particular proteins or species of nucleic acid as sites of binding has provided a significant increase in our understanding of the overall architecture and functioning of a given multi-subunit array. This has been particularly evident in studies of the ribosome (Pellegrini and Cantor, 1977), where a variety of reagents has been used to glean information about the subunit topography. Several aminoacyl tRNA species where photolabile functions have been introduced into the relevant amino acid or directly on to the tRNA have been found to function competitively at the ribosomal peptidyl transferase sites (Hsiung and Cantor, 1974; Schwartz et al., 1975; Bispink and Matthael, 1973). Identification of sites of cross-linking on photolysis has shown both ribosomal proteins and ribosomal RNA to be in the site where the 3'-end of tRNA is located. Interestingly, Schwartz and Ofengand (1978) obtained cross-linking to 16S RNA at the ribosomal P site whether or not the valyl tRNA probe had an azido function substituted in the 4-thiouridine base. Even the presence of the 4-thiouridine moiety itself (an inherently photolabile group) was found not to be necessary for cross-linking, showing that direct photoaffinity labelling between proteins and nucleic acids takes place at suitable wavelengths. Studies on the mRNA binding site in E. coli ribosomes using such direct photolysis of a poly U-ribosome complex, has shown protein S1 to be specifically involved in mRNA binding (Fiser et al., 1975; Margaritella and Kuechler, 1978) and this is confirmed using the synthetic photolabile polymer, poly(4-thiouridylic) acid (Fiser et al., 1977), although in this latter case, a number of other interactions with proteins and 16S RNA was observed.

It was generally assumed that nucleic acids cross-linked to proteins by virtue of their comparatively high absorbance properties, allowing excited electronic states to react. Antonoff and Ferguson (1978) showed, however, that in a direct photoaffinity labelling experiment with cyclic nucleotides, the action spectrum resembled protein absorbance characteristics very closely. This implies that in many direct photolabelling studies, activated amino acid residues are cross-linking to nucleic acid rather than the reverse. This highlights a drawback of the method in that there may not be any suitably disposed photolabile residues in the binding site, and such cross-linking as is detected may represent non-specific reaction. Also, competing photochemical processes may well be occurring so that the geometry of the protein and/or nucleic acid in the complex may be altered. Finally, it would be extremely difficult to determine the photochemistry occurring and allow one to maintain with any degree of certainty that non-selective and rapid insertion (criteria for true photoaffinity labelling) was taking place.

As far as photoaffinity labelling studies with nucleic acids are concerned, tRNA has been the subject of most enquiries, due to the unique occurrence of a number of different modified bases permitting specific introduction of photolabile groups at selected positions. Aside from ribosomal interactions, the interaction with the tRNA synthetases has been investigated with a number of photolabile derivatives (Budker et al., 1974; Wetzel and Söll, 1977). Since it should be possible to modify tRNA at several further positions, the entire locus of tRNA binding to synthetase could be mapped. However, there is a notable lack of studies aimed at elucidating by affinity labelling the nature of binding of other RNA or DNA species in their many complex interactions within the cell. The reason is that introduction of affinity labels into selected regions or sequences of the nucleic acid would seem to be impossible with currently available techniques. Base specific modification e.g. at cytidine residues, would appear to be a possibility and this might achieve some selectivity at less highly structured regions of the nucleic acids, but in general would appear to

be a less than useful technique, for the resulting nucleic acid would be likely to be heterogeneous to an unidentifiable degree. Cooperman (1976) has suggested that introduction of the affinity label into the protein of interest would be one approach, but this seems impracticable since in many cases it is the proteins themselves which are sought as a result of the cross-linking reaction.

The resolution of this problem is still awaited; one approach is, however, to incorporate photolabile analogues of naturally occurring nucleotides into the nucleic acid via enzymic synthesis. 5-Bromodeoxyuridine is incorporated in place of thymidine into DNA and photolysis at wavelengths greater than 300 nm (i.e. clear of the protein absorption) leads to debromination, production of free radicals and cross-linking to protein. Weintraub (1974) showed the potential of this technique when cross-linking Brd Urd-substituted DNA to a variety of histones and non-histone proteins (including RNA polymerase). Its usefulness has been confirmed in studies on the E. coli lac repressor, where, not only does a specific irreversible cross-linking of repressor to operator appear to occur on photolysis (Lin and Riggs, 1974), but mapping of the regions of operator DNA specifically cross-linked has been performed (Ogata and Gilbert, 1977).

The production of RNA species with photolabile groups contained within them presents problems since many analogues possessing base substitutions are not efficiently incorporated into RNA by RNA polymerase (Kapuler and Reich, 1972; Kumar et al., 1977), so that experiments akin to those cited above for DNA are not immediately available. On the other hand, such analogues can be incorporated at a low level when the normal nucleoside triphosphate is present as well (Kapuler and Reich, 1971) and it seems possible (as discussed in Chapter Two) that z^8Ado residues might be so incorporated into transcribed RNA species. The fact that many of the functions of heterogeneous RNA can be reproduced by homopolymeric or random RNA as synthesised by PNPase has been taken advantage of in the use

the homopolymeric poly(4-thiouridylic) acid as a photoaffinity label at the mRNA binding site of ribosomes (see above). It was the intention of the present investigation to produce RNA species containing 8-azido groups that could be employed as photoaffinity labels and to test their efficacy in different systems. The synthesis of both $(z^8A, A)_n$ and $(z^8I, I)_n$ was achieved as reported in Chapter Two, and as shown below, this type of copolymeric photolabile RNA species is able to provide important structural information in a suitable system.

Cooperman (1976) has pointed out that a high degree of substitution in the nucleic acid (e.g. in BrdUrd-substituted DNA or poly(4-thiouridylic) acid above) is likely to lead to the generation of much redundant reaction at points other than the primary contact site(s). The advantage of the PNPase-catalysed RNA synthesis is that the level of photolabile nucleotide in the polymer can be easily controlled by adjustment of the input levels of nucleoside diphosphate, so that a higher degree of specificity is likely to be achieved at a lower level of substitution.

Initially, the interaction of $(z^8A, A)_n$ with the enzyme, ribonuclease A, was investigated as a model system. This was initiated on the premise that by defining the reaction characteristics of the photoprobe with a relatively sample target, optimum conditions could be achieved more easily in experiments with (i) the relatively more complex system of *E.coli* RNA polymerase (see below) and (ii) the tissue culture conditions of interferon induction (Chapter Four).

3.1.2 Ribonuclease A

Ribonuclease A is specific in that it cleaves polyribonucleotide chains after pyrimidine residues, and hydrolyses non-pyrimidine containing polymers slowly, if at all (Richards and Wyckoff, 1971). However, an early study by Beers (1960) purported to show that A_n was hydrolysed at significant rates by high concentrations of RNase and at low salt concentrations. This view was supported by studies using a highly purified preparation (Imura *et al.*, 1965) which showed the Michaelis binding constant of A_n was at least as high as that of U_n, but that V_{max} was three orders of magnitude lower. The 2',3'-

cyclic phosphate is formed quite readily but the subsequent production of the 2'(3')-monophosphate is extremely slow. The kinetic data were confirmed (Avramova et al., 1970) and it was suggested, from a study of the reaction by ^{31}P NMR (Cozzone and Jardetzky, 1977) that only the transesterification reaction was catalysed by the enzyme. Due to a displacement of the ribose-phosphate moiety caused by accommodation of the purine base at the site thought specific for pyrimidines, it seems likely that one of the two important histidine residues is unable to complete the full in-line mechanism. These results show that ribonuclease A should be an effective model system for the assessment of $(z^8A, A)_n$ as a photoaffinity label.

3.1.3 E. coli RNA polymerase

This multi-subunit enzyme is responsible for transcription of DNA into RNA in the presence of complementary ribonucleoside triphosphates. An important question concerns the role of the different subunits in this complex synthetic reaction. Specifically, the present investigation was instigated in the expectation that a suitable photoaffinity label employed as a template for the enzyme would allow, on photolysis, the detection and isolation of the particular subunit(s) involved in template binding.

An important point of consideration here concerns the use of an RNA species to define the binding characteristics of an enzyme which is generally known to be DNA-directed in vivo. Early investigations showed that in crude preparations of the enzyme from M. luteus there appeared to be an RNA-dependent incorporation of ribonucleoside triphosphate into RNA (Nakamoto and Weiss, 1962); such activity was unlikely to be due to PNPase since this shows no such template dependency. This observation was confirmed with purified preparations of A. vinelandii RNA polymerase (Krakow and Ochoa, 1963) and shortly afterwards with both E. coli (Niyogi and Stevens, 1965) and M. luteus (Fox et al., 1964) enzymes. It appeared then that single stranded RNA could be an effective template for RNA polymerase and was probably located at the template binding site normally occupied by

native DNA . This view is strengthened by a number of studies which show that single-stranded DNA and RNA is able to inhibit transcription of a helical DNA template quite effectively (Tissières et al., 1963; Wood and Berg, 1964). More importantly, it is known that RNA polymerase can bind very efficiently to single stranded polynucleotides in what is essentially an irreversible manner, at least on the time-scale of the kinetic experiments performed (Jones and Berg, 1966), and that the order of addition of substrate and/or inhibitor is therefore of some importance. The consequence of this were first noted by Tissières et al. (1963) and investigated rather more thoroughly by Fox et al. (1965), who showed that if RNA were added to a pre-incubated RNA polymerase-DNA mixture, then no inhibition of transcription was observed. If, on the other hand, the enzyme was preincubated with RNA, then much lower transcription was found after addition of DNA to the reaction mix, i.e. inhibition was quite high. This inhibition by RNA in the presence of template has often been implicated as a cellular control of its own synthesis, and it has been conclusively shown that the gradual diminution in rate of transcription of DNA in vitro is due to accumulated RNA, either bound to the elongation complex or by some other unknown mechanism (Krakow, 1965; Maitra and Barash, 1969).

Thus, it is known that the inhibition of DNA transcription is not of a simple and reversibly competitive type, although it seems clear that RNA can bind to RNA polymerase and act as a template. Binding of nucleic acids to RNA polymerase is a complex phenomenon, depending on whether double or single stranded regions are prevalent, and whether specific promoter sites are present. Initial binding is largely electrostatic (de Haseth et al., 1978), and it seems that the affinity of the polymerase for single stranded regions, other things being equal, is much larger than for double strands, those with the least structure, e.g. U_n, being preferred. These are also the best templates amongst the RNA species; double stranded RNA is hardly transcribed.

The evidence suggests that a single stranded photolabile RNA species such as $(z^8A, A)_n$ will interact strongly with the enzyme, and might act as a template, inhibiting the transcription of DNA. Such a system would hold a degree of promise in investigating subunit topography.

The enzyme consists of two large subunits, designated β' and β, combined with a pair of smaller species, α and σ. The holoenzyme is $\beta'\beta\alpha_2\sigma$; the core enzyme, which displays both tight binding of template and high transcriptional activity, but has lost the ability to select specific promoter regions on DNA, is $\beta'\beta\alpha_2$ (Burgess, 1976).

Previous attempts to delineate the template binding interactions have been less than unequivocal. It is possible to dissociate the 4 species of subunit and, of these, only β' has displayed any DNA binding ability (Fukuda and Ishihama, 1974). Binding of $d(A\text{-}T)_n$ as a template preferentially protects the β' and β subunits against the labelling of ε-amino groups of lysine residues by fluorescamine (cited in Zillig et al., 1976), whilst the β and β' are not protected against cleavage by protease in the presence of σ subunit or template (Lill and Hartmann, 1975). Two attempts to approach the problem by photolabelling of enzyme in the presence of template were reported. Frischauf and Scheit (1973) used the synthetic deoxy polymer $(ds^4T)_n$ and inferred, from a rather poor electrophoretic analysis, that the β' subunit had been preferentially labelled. Conversely, covalent cross-linking of poly $d(A\text{-}T)_n$ to enzyme by photolysis was thought to involve either the σ or α subunit (Strniste and Smith, 1974).

It was hoped that the approach outlined below would prove of more value in positive identification of template binding sites. After completion of the work, two reports were published (using the photo-affinity technique with different probes to that described here) which essentially confirmed the conclusions arrived at by this author (Okada et al., 1978; Hillel and Wu, 1978).

3.2 Materials and Methods

3.2.1 Materials

(a) [^{14}C]-UTP (8.5mCi/mmol) and [^{14}C]-ATP (9.9 mCi/mmol) were products of Schwarz/Mann, Orangeburg, New York.

[^{14}C]-ATP (5 27 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, U.K.

(b) Glass-fibre filter discs were Whatman GF/A (2.5 cm) supplied by Whatman Ltd., Maidstone, Kent, U.K.

(c) DNA-dependent RNA polymerase (RNA nucleotidyl transferase, nucleoside triphosphate: RNA nucleotidyl transferase. E.C.2.7.7.6) was prepared by the method of Sternbach et al. (1975) and was the kind gift of Dr. V.W. Armstrong, Max-Planck Insitut für Experimentelle Medizin, Göttingen, West Germany. It had a specific activity of 1860 U/mg when measured against calf thymus DNA as template (Burgess, 1969).

(d) Sodium dodecyl sulphate was a specially pure reagent for electrophoresis supplied by BDH Chemicals Ltd., Poole, Dorset, U.K.

(e) UTP, ATP, d(A-T)$_n$, A_n and C_n were products of Boehringer Corporation (London) Ltd., U.K.

(f) Ovalbumin was supplied by Sigma (London) Chemical Co., U.K.

(g) Individual items quoted in the text were as in Section 2.2.1, and all other reagents were of analytical grade unless otherwise stated.

3.2.2 General methods

(a) Gel electrophoresis was performed using a number of different systems:

(i) Cylindrical gels were cast in glass tubes at 5% (w/v) acrylamide using the SDS-phosphate method of Weber and Osborn (1969).

(ii) Non-denaturing gels were poured in vertical slabs of 1 mm thickness at a concentration of 5% (w/v) acrylamide for the resolving gel using the high pH discontinuous system described by Maizel (1971). The slabs were topped with a 4 cm depth of stacking gel at 3% (w/v) acrylamide.

(iii) Exponential SDS 5-15% (w/v) acrylamide gels were poured in slabs (as above) using the discontinuous system of Laemmli (1970) with 3.3% (w/v) stacking gels overlaid to a depth of 4 cm.

In all systems the unpolymerised acrylamide was overlaid with n-butanol saturated water and the gels allowed to set. This gives superior interfaces between the resolving and stacking gels compared to water.

Samples were prepared as described in the text (but see Section 3.2.6), and electrophoresed toward the anode at constant current (4 mA per gel for cylinders, and 20 mA for the slab gel) until the bromophenol blue tracking dye had moved to within 1-2 cm of the bottom.

(b) Photolysis experiments were performed using the apparatus as described in Section 2.2.2 and was normally conducted at 22°. Light energy falling on the quartz cuvette containing the enzyme-inhibitor complexes was 2×10^4 erg/sec/mm^2, measured with a Hilger-Schwarz thermopile type FT. 23.1.

(c) Protein concentration was determined by the method of Lowry et al. (1951)

3.2.3 Assay of ribonuclease A

Due to difficulties experienced with older methods of ribonuclease assay, a simple, new procedure was devised. The synthetic homopolyribonucleotide C_n displays marked hypochromicity at 270 nm; the loss of this in the presence of low concentrations of ribonuclease A can be followed easily.

Typically, a solution of C_n (1-2 mM) in 0.1 M sodium dimethylglutarate, pH 7.0 (0.3 ml) in a 1 mm quartz cuvette was incubated in the thermostatted (30°) cell holder of a UV spectrophotometer. After 10 minutes, the solution of ribonuclease A (10 µl) was added, and the trace of absorbance at 270 nm against time recorded. Under these conditions, initial rates for kinetic experiments were easily measured on the linear part of the curve. It was found that extreme care in the handling of the C_n stock solution was necessary to avoid contamination and rapid hydrolysis. All solutions were prepared with sterilised double distilled water, and gloves were used to keep skin nucleases from coming into contact with glassware.

3.2.4 Assay of RNA polymerase

To a small assay tube the following reagents were added by micropipette:

0.16 M bicine (or tricine), pH 8.0, 0.8 M KCl (25 µl)

0.01M $MgCl_2$ (10µl)

$d(A\text{-}T)_n$ (10 $O.D_{260}$/ml, 10 µl)

0.01M UTP (10 µl)

0.01M [^{14}C]-ATP (1.76×10^3-2.175×10^4 cpm/nmole, 10 µl)

Water (sufficient to bring volume to 100 µl when enzyme added).

This was vortexed, then incubated at 37° for 10 minutes. After addition of enzyme solution (total volume now 100 µl) with mixing, incubation was performed at 37° with suitable aliquots withdrawn at intervals and dispensed on to glass fibre filter discs. These were dried under an infra-red lamp and suspended in stirred 5% TCA for 15 minutes to precipitate RNA and wash off mononucleotides. After a second wash in 5% TCA (15 minutes), the filters were passaged through absolute ethanol (5 minutes) and diethyl ether (5 minutes), dried under the lamp and counted in toluene scintillant (10 ml). In early experiments, [^{14}C]-UTP was used as the radioactive nucleotide at 18,650 cpm/nmole.

In some experiments, the reaction was initiated by addition of the nucleoside triphosphates. Additions of inhibitors and other variations in procedure are as noted in the text, and in legends to the tables and diagrams.

3.2.5 Inactivation of enzymes by $(z^8A, A)_n$

(a) Ribonuclease A

Enzyme (0.2 µg) was incubated with $(z^8A, A)_n$ (Ado: z^8Ado = 6.5:1, 0.715 mM) at 4° in sodium dimethylglutarate buffer, pH 7.0 (0.1 M, 0.1 ml) for 10 minutes and then subjected to photolysis at 22°, as described above, for various periods. After each photolysis, an aliquot (10 µl) was withdrawn and assayed for activity in the C_n assay. A variety of protection and scavenging experiments was performed, and these are described in the text where relevant.

(b) RNA polymerase

Incubations of enzyme (4-20 μg) with $(z^8A, A)_n$ (Ado: z^8Ado = 6.5:1) at various concentrations to form binary complexes were performed for 10-15 minutes at 37° prior to photolysis. Incubations were conducted in 0.1 ml total volume in 0.04 M bicine (or tricine), pH 8.0, 0.05 M potassium chloride, 0.01 M magnesium chloride. These low salt conditions are conducive to optimum formation of binary complexes. After photolysis for varying periods at 22° (as described above), aliquots (10 μl) were withdrawn and dispersed into assay solution (minus the nucleoside triphosphates) kept on ice, to give a total volume of 80 μl. Initiation of the assay was preceded by 15 minutes incubation at 37° to allow equilibrium of substrates, before addition of the mixed UTP/[^{14}C]-ATP solution (20 μl). As for ribonuclease, a variety of conditions involving protection experiments etc. were investigated, these being detailed in the relevant legends to tables and diagrams.

3.2.6 Determination of RNA polymerase subunits cross-linked by photolysis in the presence of $(z^8A, A)_n$

Enzyme was incubated with $(z^8A, A)_n$ and photolysed for 2.5 minutes under the conditions described above. The quantities used in different experiments are detailed in the legends to the relevant diagrams, but a typical incubation involved RNA polymerase (16 μg) and $(z^8A, A)_n$ (41.6 μg) in buffer (0.2 ml). After photolysis, the solution was cooled to 4° and an equal volume of ice-cold TCA (20% w/v) added, followed by centrifugation at 4° in a bench centrifuge for 20 minutes. The supernatant was withdrawn, cold acetone (0.2 ml) was added with vortexing and the solution centrifuged once again for 20 minutes. The supernatant was discarded, the pellet dried in a gentle stream of air and taken up in protein dissolution buffer (0.08 ml). (Protein dissolution buffer comprises 0.05 M tris-HCl, pH 9.0, 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. After heating to 100° for 2 minutes, the sample was prepared for electrophoresis by addition of sucrose (40% w/v, 0.02 ml) and bromophenol blue (0.2%

w/v, 0.5 µl) and electrophoresed in 5-15% gradient gels as described above. Areas containing cross-linked high molecular weight products (location was determined by staining a control gel in Coomassie Brilliant Blue R-250, and was found to comprise the top 0.5 cm) were excised and sliced into small pieces. These were then dispersed into water (0.8 ml) by means of a number of strokes of a loose-fitting glass homogeniser of the Douncetype, followed by shaking overnight at 37° C to extract protein. The polyacrylamide was spun down in the bench centrifuge and the supernatant withdrawn. A further extraction into water was performed and the supernatants combined (ca. 1 ml total). Ovalbumin (25 µg) was added to the solution and, after cooling, protein was precipitated by addition of ice-cold trichloroacetic acid to 10% final concentration and isolated by centrifugation and washing procedures as above.

The dried pellet was taken up in tris-HCl, pH 6.8 (0.0625 M, 0.05 ml) and incubated with ribonuclease A (10 µg) for 3 hours at 37° C, followed by addition of SDS (10% w/v, 0.01 ml) and ribonuclease (10 µg) for a further 3 hour period at 37° C. Preparation for gel electrophoresis was similar to that already described, the solution being made up with protein dissolution-buffer to 0.08 ml with an extra addition of 2-mercaptoethanol (0.001 ml), heated to 100° C for 2 minutes etc.

3.3 Results and Discussion

3.3.1 Ribonuclease A as a model system

In order to follow the effects of photolysis of ribonuclease A in the presence of $(z^8A, A)_n$, a simple and rapid assay was required. Imura et al. (1965), in their study of the hydrolysis of A_n, used a standard RNA precipitation assay involving uranyl acetate in perchloric acid, with measurement of the absorbance of the mononucleotide-containing supernatant. In our hands this assay proved to be highly unreliable in terms of reproducibility and low sensitivity to changes in the concentration of substrate U_n under subsaturating conditions.

It is apparent that under the highly acidic conditions reported, quantitative precipitation is not observed and that much more dilute acid needs to be used (Dickman and Trupin, 1959). As the method is only suitable for quantitation of uridylic acid monomers, it will clearly give unreliable results for A_n hydrolysis anyway. Thus the kinetic parameters deduced by Imura et al. (1965) must be treated with some reserve. Lepoutre et al. (1963) showed that precipitation methods of assay such as these were not trustworthy because co-precipitation of monomers and polymers was found to occur with a number of precipitants.

It seemed likely that a simpler assay could be devised based on the original observations of RNA hyperchromism on ribonuclease hydrolysis (Kunit , 1946). C_n is an excellent substrate for ribonuclease A and as such was used as a substrate in a precipitation type assay (Zimmerman and Sandeen, 1965). In the present study, it was found that direct observation of the rate of increase in absorbance of C_n at 270 nm in the presence of ribonuclease served as a rapid and reproducible indication of enzyme activity. The use of 1 mm path-length cells allowed a range of C_n concentrations to be used since the absorbance at 270nm falls in the normal 0-2 range. The utility of the technique is increased by the ready availability of sensitive UV spectrophotometers with offset and scale expansion facilities. This widens further the range of conditions over which direct monitoring is possible, and makes the assay as sensitive as any precipitation technique. Commonly, assays were performed here with ca. 10 ng of ribonuclease A, but it was possible to reduce this by a factor of 1000 and still monitor the hyperchromism of C_n.

A study of C_n hydrolysis in 0.1 M dimethylglutarate buffer, pH 7.0, at 30° showed a straight line graph when plotted according to the double reciprocal method of Lineweaver and Burk (1934), and yielded a Michaelis constant (in mononucleotide units) of 0.50 mM (Figure 3.2). Under identical conditions, no hydrolysis of A_n of $(z^8A,A)_n$ was observed. On the other hand $(z^8A,A)_n$ proved to be an effective competitive inhibitor of ribonuclease-catalysed C_n hydrolysis (Figure 3.2) with a graphically determined inhibition constant of 0.512 mM.

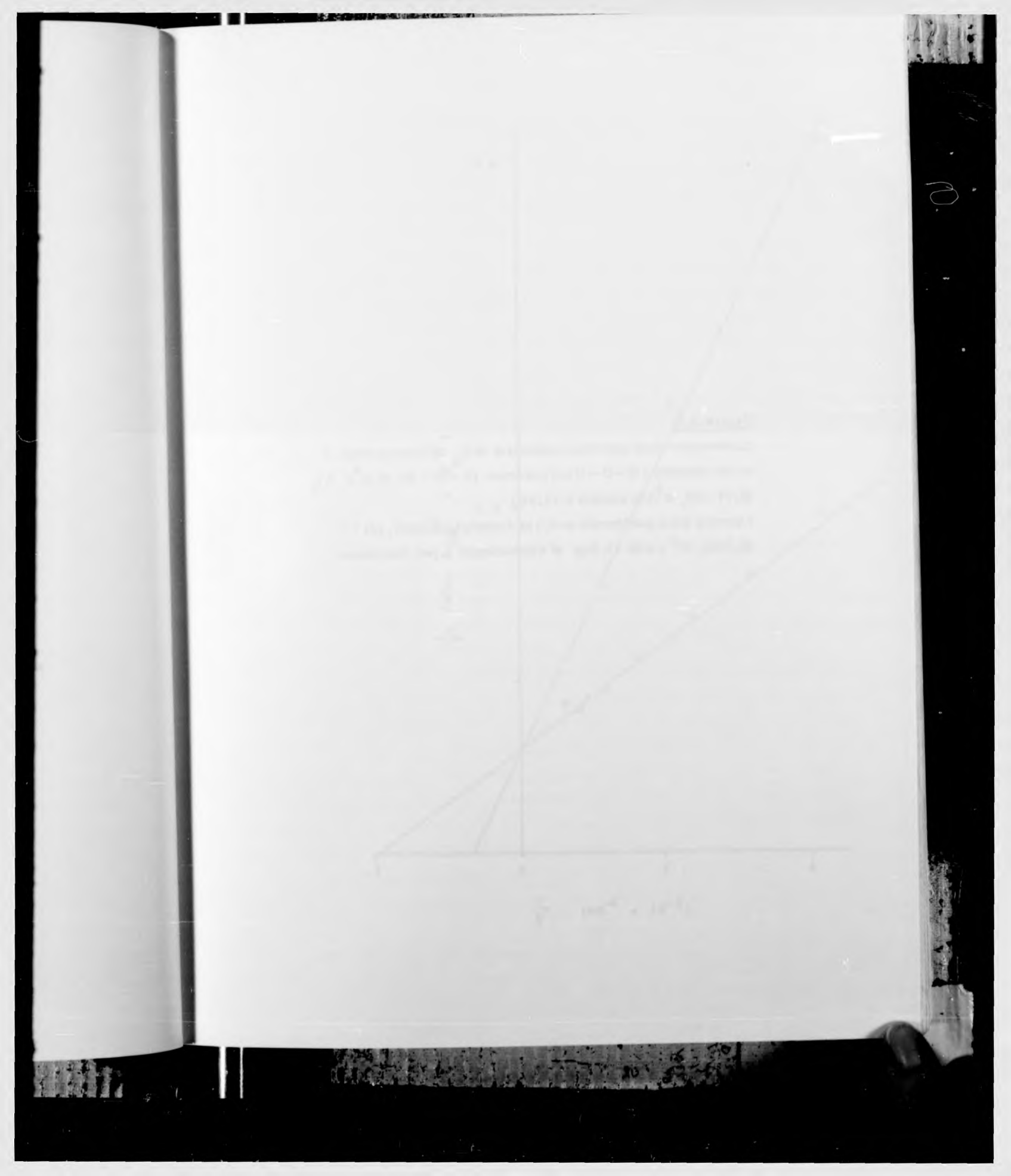
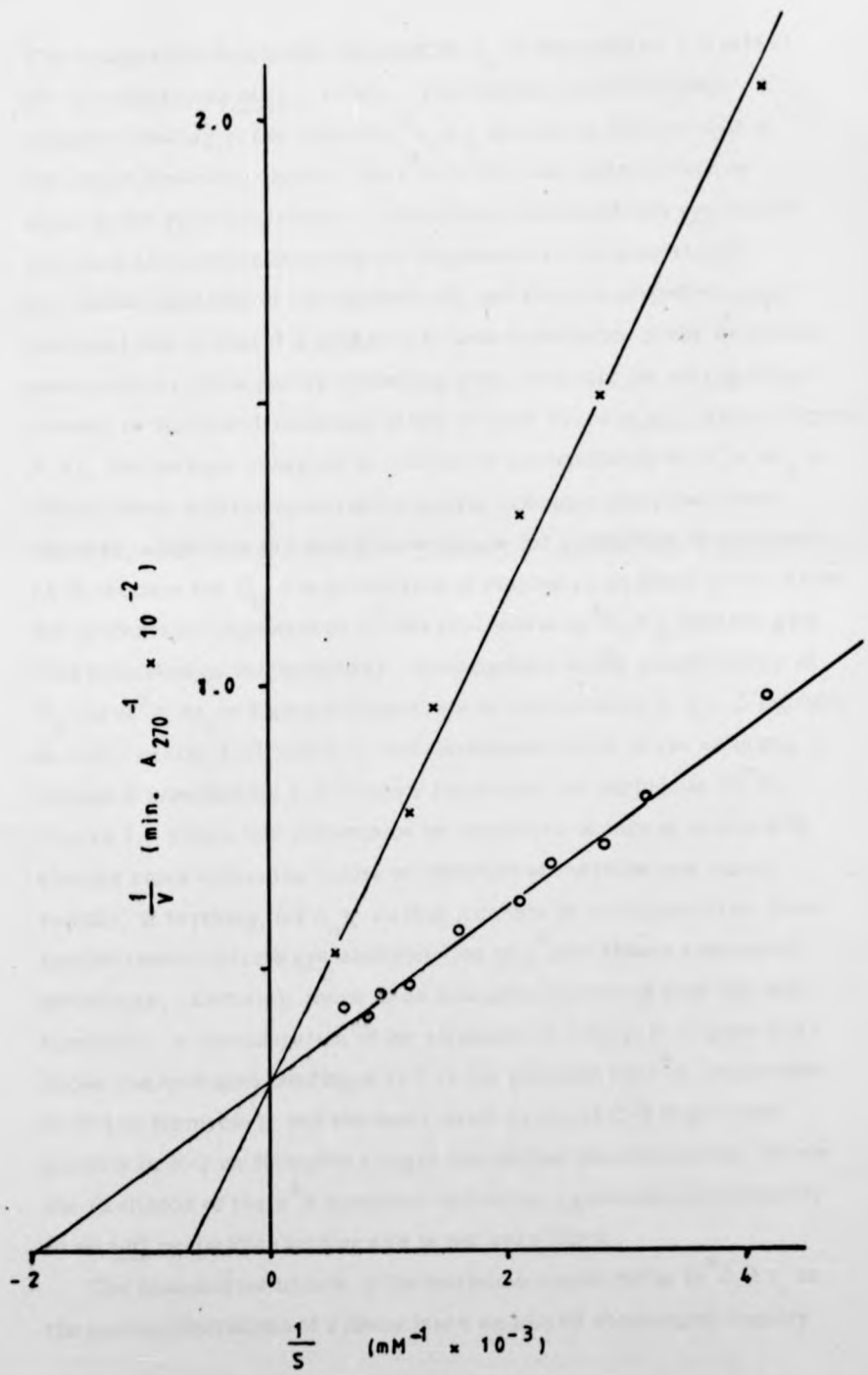


Figure 3.2

Lineweaver-Burk plots for hydrolysis of C_n by ribonuclease A in the absence (O – O – O) or presence (X – X – X) of $(z^8A, A)_n$ (0.91 mM, z^8Ado content = 13.4%).

Kinetics were performed in 0.1 M dimethylglutarate, pH 7.0 (0.3 ml, 30°) with 12.5 ng of ribonuclease A per incubation.


2.0
1.0
$\frac{1}{V}$ (min. A_{270}^{-1} x 10^{-2})
-2
0
2
4
$\frac{1}{S}$ (mM^{-1} x 10^{-3})

The comparable Michaelis constant for A_n is reported as 1.3 mM at pH 7.0 (Avramova et al., 1974). The reasons for the slightly enhanced binding in the case of $(z^8A, A)_n$ may lie in the fact that in the single stranded polymer, the z^8Ado residues are expected to exist in the syn conformation. It has been postulated that syn purine residues are conformationally not dissimilar to the natural anti pyrimidine substrate of ribonuclease A, and that a hydrogen-bonding potential akin to that of a hybrid U/C base is reflected in the increased susceptibility of the purine containing poly formycin (an all syn single strand) to the hydrolytic action of the enzyme (Ward et al., 1969) (Figure 3.3). We have not observed an increased susceptibility of $(z^8A, A)_n$ to ribonuclease with the spectrophotometric technique described here; however, while this is a sensitive technique for production of monomers, as in the case for C_n, the production of oligomers as would be the case for preferential digestion at z^8Ado residues in $(z^8A, A)_n$ will not give rise to marked hyperchromicity. Investigation of the susceptibility of A_n and $(z^8A, A)_n$ to high concentrations of ribonuclease A (ca. 5 mg/ml) in 0.017 M tris-HCl, pH 7.2, was performed by tlc of the solutions in solvent F (see Section 2.2.2) after incubation for periods at 37°C. Figure 3.4 shows that conversion to oligomers occurs at essentially similar rates within the limits of detection of the technique (more rapidly, if anything, for A_n), so that it cannot be concluded from these limited results that the syn conformation of z^8Ado affects hydrolytic sensitivity. Certainly there is no dramatic change as seen for poly formycin. A consideration of the structure of formycin (Figure 3.3) shows that hydrogen bonding at C-9 is not possible for z^8A (equivalent to N-1 on formycin), and the bulky azido group at C-8 (equivalent position to N-2 on formycin) might destabilise the interaction. Hence the likelihood of the z^8A monomer achieving a good complementarity to an anti pyrimidine binding site is not very likely.

The competitive nature of the inhibition displayed by $(z^8A, A)_n$ at the low concentrations of ribonuclease employed encouraged enquiry

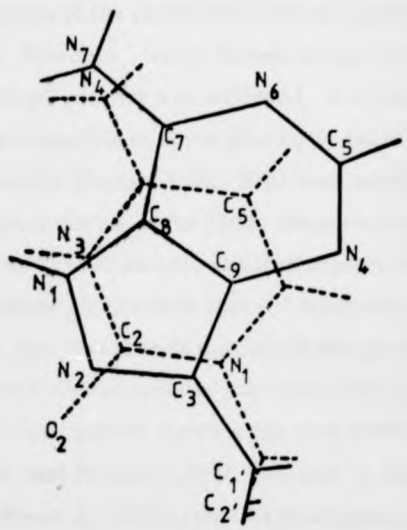
Figure 3.3

Correspondence between the syn conformation of the purine, formycin and a hybrid structure of uracil and cytosine bases (used by Ward et al. (1969) as an explanation for the high susceptibility of poly formycin to ribonuclease A hydrolysis)

Figure 3.4

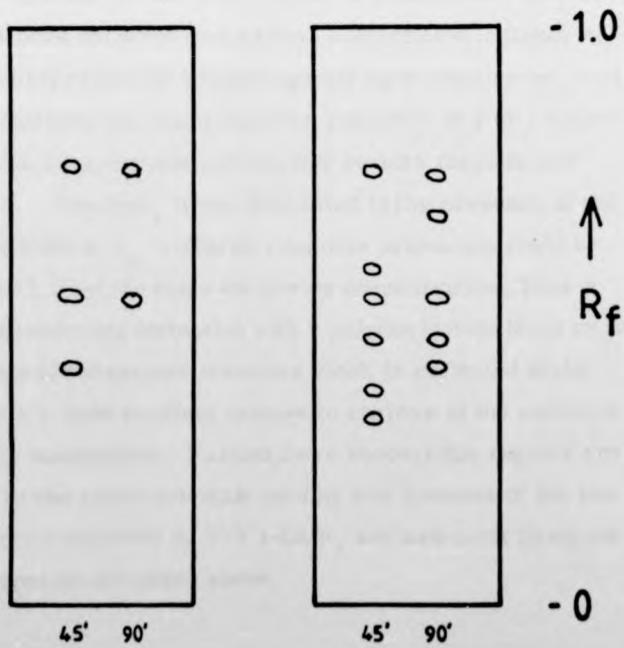
Thin layer chromatographic evidence for the resistance of (a) A_n and (b) $(z^8A, A)_n$ to ribonuclease A hydrolysis. Equal quantities of polyribonucleotide and enzyme (0.5 mg) in 0.017 M tris-HCl, pH 7.2 (0.1 ml) were incubated at 37° for the stated periods; aliquots were spotted on to silica tlc plates which were developed in solvent F.

3.3


N7
N4
N6
C7
C5
C5'
C8
N3
C9
N4
N1
C2
N2
N1
C3
O2
C1'
C2'
F

3.4

a) b)


-1.0
Rf
-0
45' 90'
45' 90'

into the characteristics of the polynucleotide as a photoaffinity label. Initial investigation, however, led to disappointing results since although inactivation of enzyme was achieved, it appeared that the enzyme itself was susceptible to some photolytic inactivation under the conditions employed (Figure 3.5). This was surprising in view of the filtered characteristics of the light, the wavelength of which should have been clear of the protein absorption peak at 280 nm (see inset, Figure 2.17). However, it is known that UV inactivation of ribonuclease takes place readily, and that broken disulphide linkages may be the primary cause for this loss of activity (McLaren and Luse, 1961). The absorption spectrum of cystine extends out to wavelengths greater than 300 nm (Beaven and Holiday, 1952), so that it appears probable that native ribonuclease A, which contains four cystine linkages, is particularly sensitive to photolytic inactivation at these long wavelengths. In support of this, Sawada (1975) used light of 334 and 365 nm to partially inactivate the enzyme in the presence of the inhibitor 2'(3')-4-thiouridylic acid, and presented evidence to suggest that the site of cross-linking was at cystinyl residues, which occur outside the active site and explains the variable degree of inactivation achieved.

It might have been expected that a known competitive inhibitor of ribonuclease would protect the enzyme against light inactivation, and indeed some protection was observed in the presence of 2'(3')-UMP at 2 mM concentration, in agreement with earlier results (Sawada and Ichimura, 1967). However, it was found that in the presence of the polyribonucleotide inhibitor A_n, virtually complete protection could be achieved (Figure 3.5) at the same nucleotide concentration. Thus it would appear that complex formation with a polynucleotide leads to an enhanced stability of the enzyme structure which is reflected in the inability of near UV light to effect damage to regions of the molecule that are normally susceptible. Further, these susceptible regions are probably distal to the mononucleotide binding site because of the low degree of protection afforded by 2'(3')-UMP, and are most likely the disulphide linkages as discussed above.

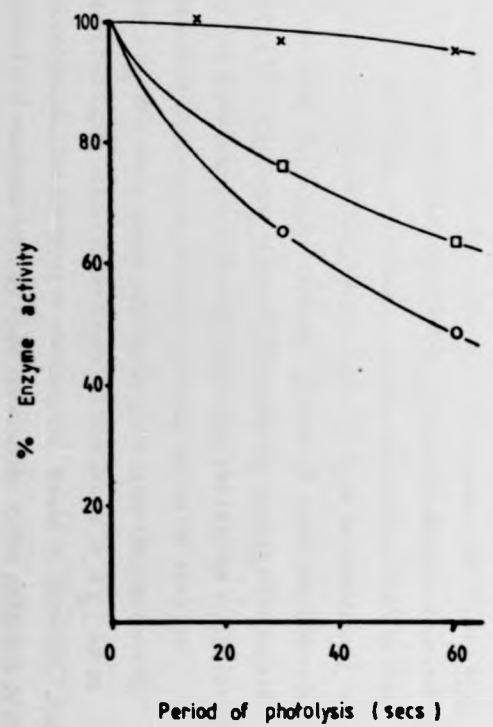
Figure 3.5

Inhibition of ribonuclease A activity by photolysis. Conditions of photolysis together with light intensity are given in Sections 3.2.2 and 3.2.5a. The pyrex/soda glass filter combination was used as a filter (see inset, Figure 2.17). Enzyme alone (O – O – O), enzyme +2 mM 2'(3')-UMP (□–□ –□), enzyme +2 mM A_n (X – X – X)

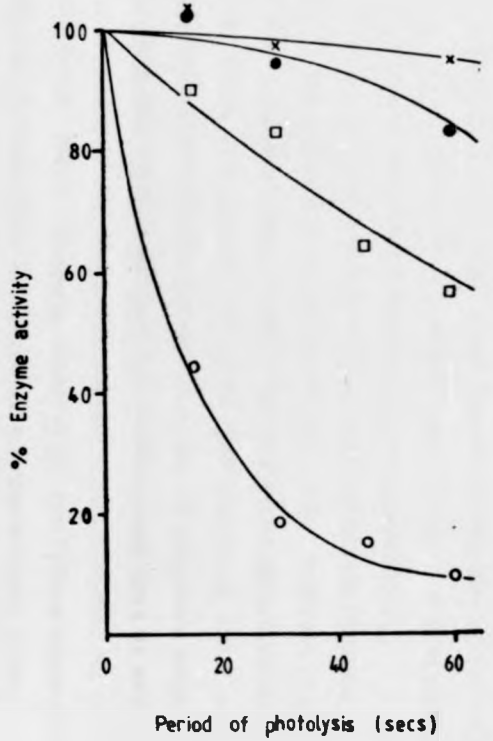
Figure 3.6

Inhibition of ribonuclease A activity by photolysis in the presence $(z^8A, A)_n$ (sample containing 13.4% z^8Ado). Conditions were as described in Figure 3.5. Enzyme + 0.715 mM $(z^8A, A)_n$ (O – O – O), enzyme +0.715 mM $(z^8A, A)_n$ + 2 mM 2'(3')-UMP (□–□ –□), enzyme + 0.8 mM A_n (●– ●– ●), enzyme + 0.715 mM prephotolysed $(z^8A, A)_n$ (X – X – X).

3.5


100
80
60
40
20
0
20
40
60
% Enzyme activity
Period of photolysis (secs)

3.6


100
80
60
40
20
0
20
40
60
% Enzyme activity
Period of photolysis (secs)

On consideration, it seems likely that the polynucleotide inhibitor maintains the integrity of the enzyme molecule by a degree of binding interaction with amino acid residues outside the active site, leading to a more rigid conformation. The effect cannot be explained by filtering of the incident light, since the optical densities of 2'(3')-UMP and A_n at the concentrations used are very similar . It is of interest to review what other evidence exists for such interactions.

In terms of specific binding sites for mononucleotide residues, it is clear that only one exists (Hummel et al., 1961; Anderson et al., 1967). On the other hand, while 2'CMP was a competitive inhibitor of both RNA hydrolysis and 2'3'-cyclic CMP hydrolysis, the K_i value determined in RNA hydrolysis was an order of magnitude larger, suggesting that the RNA was perhaps bound at other sites as well as the main catalytic site, and hence was less easily competed out (Nelson and Hummel, 1961). Recently, White et al. (1977) have observed the inhibition of ribonuclease by a series of oligonucleotides. From competition experiments they have inferred that in the inhibitor Aps^4U the purine residue does not occupy the site occupied by the purine of the substrate UpA, nor does the phosphate group occup the normal phosphate binding site. Finally, specific modification by 3-ethoxy-2-keto-butanal of the arginine residues of ribonuclease shows that altered residues outside the active site can affect the activity of the enzyme toward RNA more than that towards 2',3'-cyclic CMP (Iijima et al., 1977). It was suggested that cationic groups not directly involved with the active site might play a role in unstacking bases in RNA by interacting with the unstacked conformation. It must be understood, however, that such extra binding as exists in the case of polyribonucleotides is of quite a low magnitude, since highly specific binding sites outside the active site would almost certainly mean that the enzyme would act via a processive mechanism and this is known not to be the case.

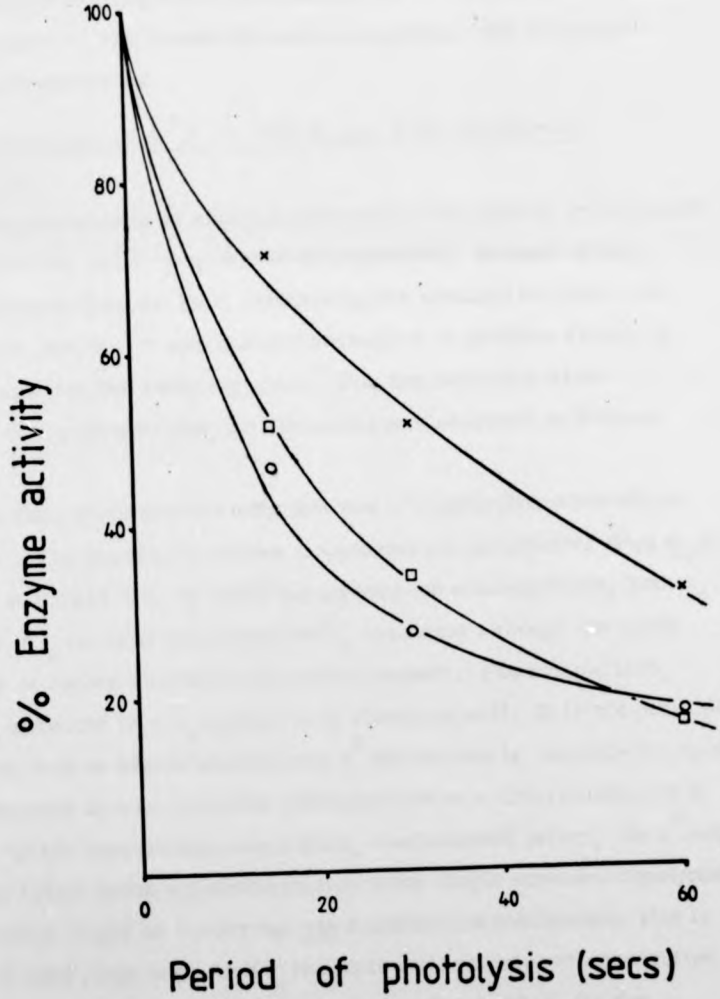
Since A_n efficiently protected the enzyme from the inactivating effect of the light, it was to be expected that the $(z^8A, A)_n$, as an efficient competitive inhibitor of the enzyme, would do likewise. Any inactivation of enzymic activity upon irradiation in the presence of the copolymer would thus be due to photolabelling. Figure 3.6 shows that

the enzyme is very rapdily inactivated (in 30 seconds) when photolysed with 0.715 mM $(z^8A, A)_n$ in 0.1 M dimethylglutarate, pH 7.0. A pre-photolysed sample of the polymer produces no inactivation of the enzyme when further photolysed, confirming the observations with A_n as expected. Lower concentrations of A_n than that in Figure 3.5 give a slightly reduced protecting effect: this may be connected with the slightly higher dissocation exhibited by A_n compared to $(z^8A, A)_n$ as discussed above. The protection to inactivation afforded by 2'(3')-UMP in the presence of $(z^8A, A)_n$ is also shown in Figure 3.6. That the degree of inactivation appears to return to the levels expected in the presence of 2'(3')-UMP alone shows that there is specific competition for the catalytic site and that this is sufficient to dispel most of the photo-catalysed inhibition by the polymer.

These results proved the effectiveness of the synthesised polyribonucleotides as photoaffinity labels. The specific nature of the inactivation of the enzyme was further probed by performing experiments with scavenging molecules which preferentially react with the activated nitrenes generated. The results of scavenging experiments are shown in Figure 3.7. A reduction of the percentage inactivation to control levels would signify one of two possibilities. Either inactivation was occurring by "pseudo" photoaffinity labelling (Ruoho et al., 1973) as discussed in Chapter One, or the scavenger was itself occupying the active site and protecting the enzyme by competition with the polyribonucleotide. Neither of these eventualities was observed with glycine at two concentrations, confirming the conclusion that specific photolabelling was taking place. The slight reduction in inactivation observed can be accounted for if one realises that at a concentration of 0.715 mM of $(z^8A, A)_n$, only ca. 60% of the ribonuclease is bound, assuming equilibrium has been attained (and using a K_i value of 0.512 mM). The rapid reaction of scavenger with unbound polynucleotides would serve to reduce the level of inactivation, since the shift in equilibrium brought about by a diminution in the concentration of non-covalently bound E.I complex would only serve to produce E.I* complexes (where I* represents a polynucleotide already reacted with scavenger).

Figure 3.7

Effect of glycine scavengers on photolytic inactivation of ribonuclease A by $(z^{8}A, A)_n$. Conditions were as described in Figure 3.5. Enzyme + 0.715 mM $(z^{8}A, A)_n$ (O − O − O), enzyme + 0.715 mM $(z^{8}A, A)_n$ +5 mM glycine (□ − □ − □), enzyme + 0.715 mM $(z^{8}A, A)_n$ + 10 mM glycine (X − X − X).


100
80
60
40
20
% Enzyme activity
0
20
40
60
Period of photolysis (secs)

A number of tryptic hydrolysis experiments on these modified enzyme molecules were performed, but the maps bore little relation to the maps of native ribonuclease. This is obviously a consequence of the indiscriminate nature of nitrene insertions and reinforces the view that fine structural mapping by photoaffinity labelling is not a valid technique (see Section 3.1.1): rather its chief value lies in the delineation of topographical relationships of subunits, etc. In the following section, the results of such an approach with RNA polymerase are presented.

3.3.2 Interaction of $(z^8A\ A)_n$ with E. coli RNA polymerase

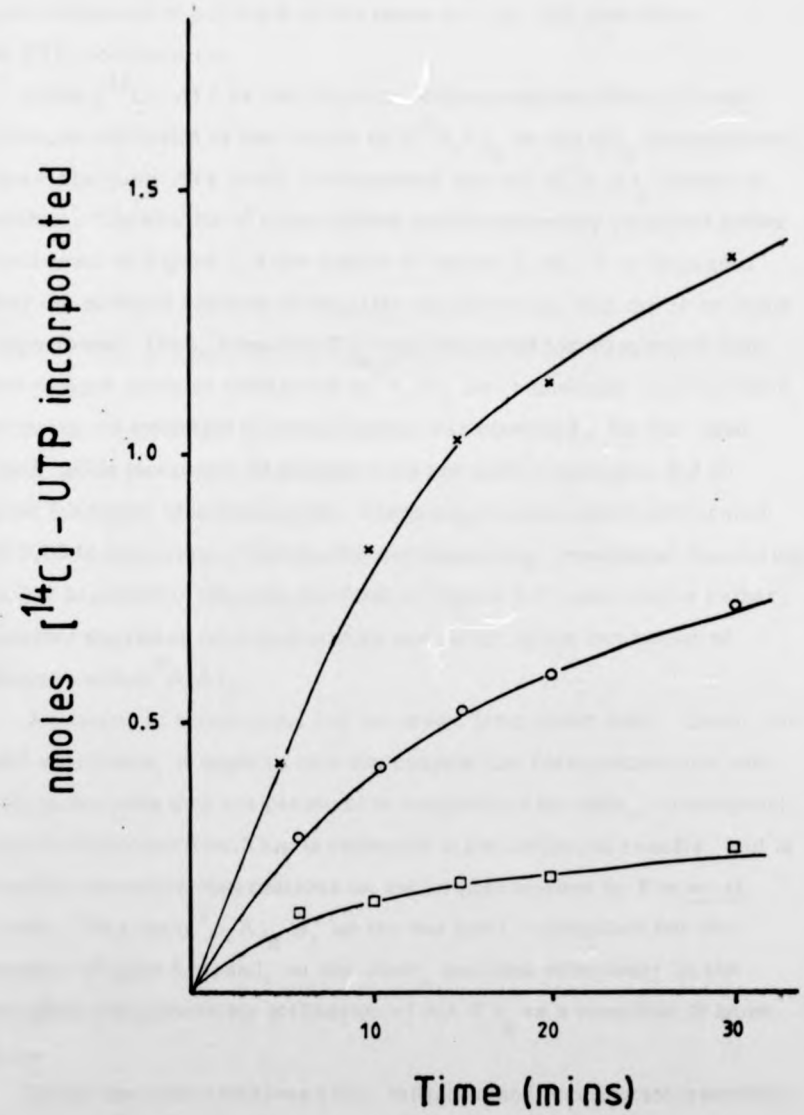
(i) Kinetics

The standard assay of RNA polymerase activity usually incorporates a thiol reducing agent, e.g. 5 mM dithiothreitol. Because of the observations in Chapter Two, concerning the reaction of thiols with 8-azido nucleotides, it was deemed necessary to perform assays in the absence of added reducing agent. For the relatively short duration of the incubations, no difference was observed in enzyme activity.

It has been found that for efficient use of a polyribonucleotide as template, it is necessary to have manganous ion as cofactor (Fox et al., 1964). In Figure 3.8, at 4 mM manganous ion concentration, both A_n and $(z^8A.A)_n$ serve as templates for U_n synthesis although the azido polymer is rather less efficient in this respect. For comparison, $d(A\text{-}T)_n$-directed $(A\text{-}U)_n$ synthesis is shown as well. It is not possible from this data to decide whether the z^8Ado residue is actually accepted by the enzyme as a template for incorporation of a Urd residue (via a Watson-Crick base pairing step) since, as discussed before, the z^8Ado probably exists in the syn conformation in the single stranded copolymer. U_n synthesis might be occurring via a reiterative mechanism; this is known to take place with $(U\text{-}X)_n$ templates where only two consecutive Urd residues appear to be necessary for synthesis of A_n (in the absence of any other nucleoside triphosphate) (Adman and Grossman, 1967).

Figure 3.8

Transcription of $(z^8A, A)_n$ by E. coli RNA polymerase. Incubations were performed at 37° as described in Section 3.2.4, using enzyme at 40 μg/ml, 0.05 M potassium chloride, 0.04 M bicine, pH 8.0. 4 mM $MnCl_2$ and templates $d(A\text{-}T)_n$ (0.147 mM, X − X − X), A_n (0.1 mM, O − O − O) or $(z^8A, A)_n$ (0.14 mM, □ −□ −□ ; sample containing 13.4% z^8Ado) in a total volume of 0.1 ml.


nmoles [14C] - UTP incorporated
1.5
1.0
0.5
10
20
30
Time (mins)

$(z^8A, A)_n$, when added to the incubation mixture with $d(A\text{-}T)_n$, was found to be a good inhibitor of $d(A\text{-}T)_n$-directed transcription (Figure 3.9) even though the observed incorporation of [^{14}C]-UTP would include $(z^8A, A)_n$-directed synthesis of U_n as well. Using equimolar concentrations of polynucleotides there is a ca. 50% reduction in UTP incorporation.

Using [^{14}C]-ATP as the indicator of transcription allows a more accurate reflection of the effects of $(z^8A, A)_n$ on $d(A\text{-}T)_n$ transcription, since obviously ATP is not incorporated into any $(z^8A, A)_n$-directed product. The results of experiments performed under identical buffer conditions to Figure 3.9 are shown in Figure 3.10. It is apparent that the order of addition of template relative to the enzyme is of some importance. Thus, when $d(A\text{-}T)_n$ was incubated for 10 minutes with the enzyme prior to addition of $(z^8A, A)_n$ and nucleoside triphosphates, virtually no inhibition of transcription was observed. On the other hand, prior incubation of enzyme with the azido copolymer led to good inhibition of transcription. These experiments were performed in 0.05 M potassium chloride; the corresponding results for incubation in 0.2 M potassium chloride, depicted in Figure 3.11, show that a rather smaller degree of inhibition occurs even after prior incubation of enzyme with $(z^8A, A)_n$.

A number of conclusions can be drawn from these data. Under low salt conditions, it appears that the enzyme can form complexes with polynucleotides that are resistant to competition by other, subsequently added polynucleotides. This is reflected in the inhibition results, and is similar to earlier observations on such a phenomenon by Fox et al. (1965). Thus the $(z^8A, A)_n$ is, on the one hand, a template for the enzyme (Figure 3.8) and, on the other, can bind effectively in the template site, preventing utilisation of $d(A\text{-}T)_n$ as a template (Figure 3.9.

Under low salt conditions also, initiation and termination reactions do not occur so readily (Maitra and Barash, 1969). Because the assay as performed represents mainly the elongation phase of the reaction,

Figure 3.9

Inhibition of $d(A\text{-}T)_n$ transcription in the presence of $(z^8A, A)_n$. Incubations were performed as above for $d(A\text{-}T)_n$ in the absence (X – X – X) and presence (O – O – O) of $(z^8A, A)_n$ (0.14 mM, sample containing 13.4% z^8Ado). Enzyme was added last to the incubation.

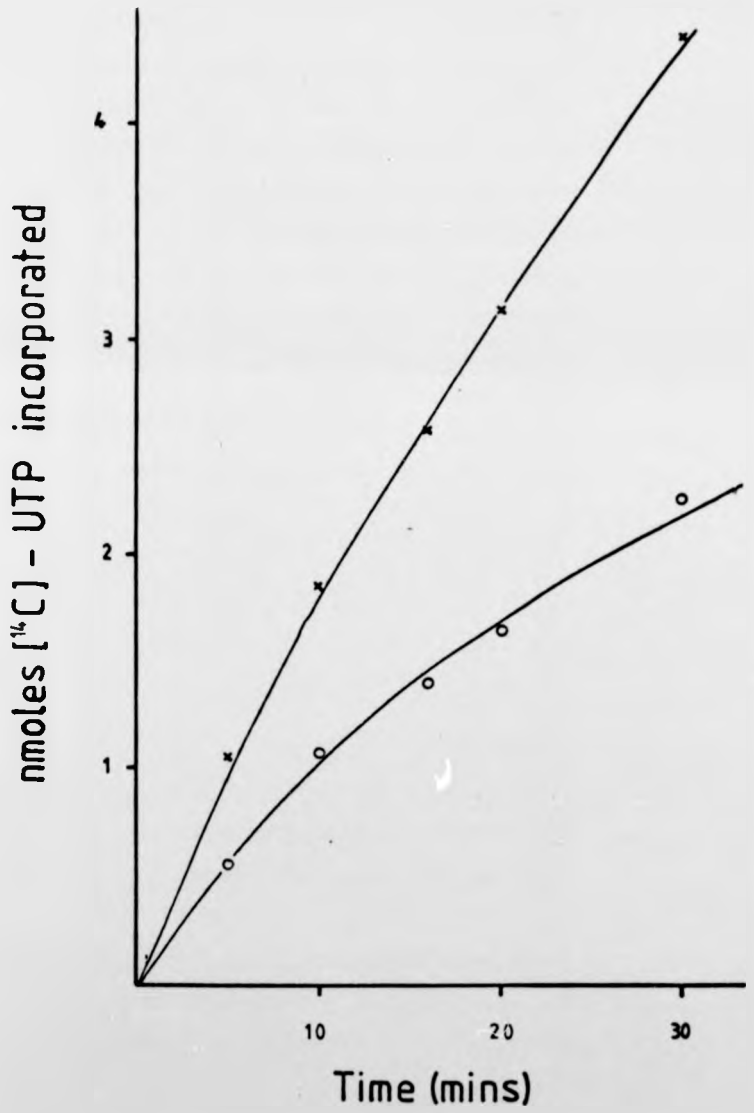

nmoles [14C] - UTP incorporated
4
3
2
1
10
20
30
Time (mins)

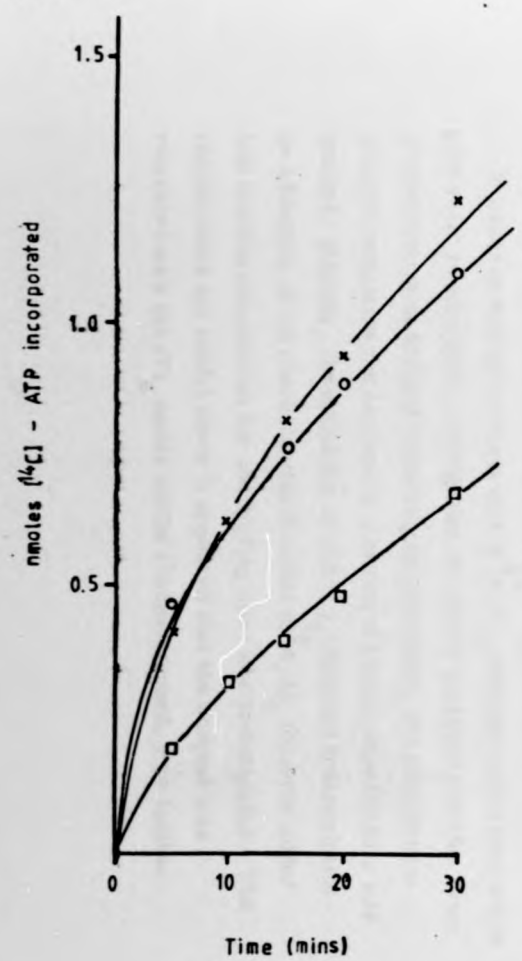
Figure 3.10

Effect of "order of addition" of $(z^8A, A)_n$ on $d(A\text{-}T)_n$ transcription by RNA polymerase at 0.05 M potassium ion. Incubations were performed in a similar fashion to those of Figure 3.8, except that incorporation of [^{14}C]-ATP was monitored, enzyme was added to the mixture of reagents prior to the nucleotides, and 10 mM Mg^{2+} substituted for Mn^{2+}. The kinetics of transcription of $d(A\text{-}T)_n$ (0.147 mM) alone (X − X − X), after 10 minutes incubation with enzyme prior to addition of $(z^8A, A)_n$ (93.5 μM) and nucleoside triphosphates (O − O − O), and after 10 minutes of prior incubation of enzyme with $(z^8A, A)_n$ (93.5 μM) before addition of the $d(A\text{-}T)_n$ (□ − □ − □) and nucleoside triphosphates are displayed.

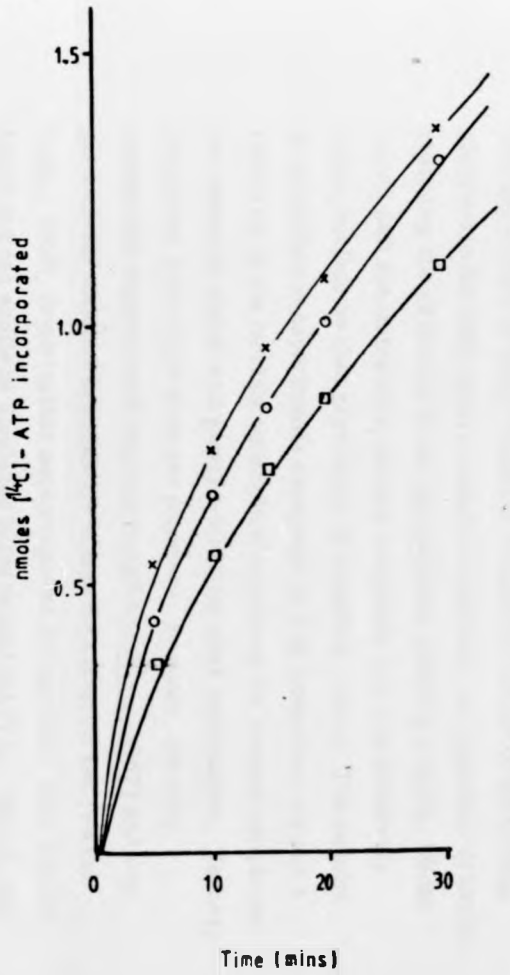
Figure 3.11

Effect of "order of addition" of $(z^8A, A)_n$ on $d(A\text{-}T)_n$ transcription by RNA polymerase at 0.2 M potassium ion. The reaction conditions were identical to Figure 3.10 except for the presence of 0.2 M K^+ throughout. $d(A\text{-}T)_n$ + enzyme (O − O − O), $d(A\text{-}T)_n$ pre-incubated with enzyme (10 minutes) before $(z^8A, A)_n$ addition (X − X − X), $(z^8A, A)_n$ pre-incubated with enzyme (10 minutes) before $d(A\text{-}T)_n$ addition (□ − □ − □).

3.10


1.5
1.0
0.5
nmoles [14C] - ATP incorporated
0
10
20
30
Time (mins)

3.11


1.5
1.0
0.5
nmoles [14C]- ATP incorporated
0
10
20
30
Time (mins)

it can be inferred that the stable binary complex with $(z^8A, A)_n$ is maintained as a stable ternary complex (with U_n) in the elongation phase. The importance of these results is the specific nature of the binding observed under these binary complex conditions, an important criterion regarding the relevance of any photoaffinity labelling results. Under higher salt concentrations, binary complexes are still relatively stable, but less so than previously (Richardson, 1966). The results of the inhibition experiments assayed at 0.2 M potassium ion are a reflection of the fact that with helical templates the ternary complexes are extemely stable with good re-initiation after termination. Ternary complexes with single stranded polymers, however, are very susceptible to premature termination (Maitra et al., 1967) and are particularly sensitive to inhibitors of free RNA polymerase (Walter et al., 1967), implying that such templates are probably only loosely bound and can easily be displaced by the helical $d(A\text{-}T)_n$. Hence, the inhibition is not marked under these conditions. In subsequent photo-labelling experiments, incubations were performed at low salt concentrations (0.05 M potassium ion) so that good binary complex formation was observed with the $(z^8A, A)_n$. Assays of enzymic activity were, however, performed at 0.2 M potassium ion, since then any genuine covalent inactivation of the enzyme would not be confused with inhibition due to non-dissociation of stable, but non-covalently associated enzyme-$(z^8A, A)_n$ complexes.

In order to further confirm that $(z^8A, A)_n$ polyribonucleotides would give valid results upon investigation of subunit specificity in the event of inactivation of enzyme occurring on photolysis, the competition between templates for enzyme in a series of kinetic experiments was studied. Initially, the inhibition of $d(A\text{-}T)_n$-directed transcription as a function of the concentration of added $(z^8A, A)_n$ (enzyme added last to allow competition for the binding site) was investigated. The results were not useful since it appeared that the enzyme was saturated with $d(A\text{-}T)_n$ under all the conditions used. Any further

reduction in concentration of $d(A\text{-}T)_n$ would have required much hotter [^{14}C] nucleoside triphosphates than were available, in order to follow the transcription reaction. The reasons for this behaviour are probably that $d(A\text{-}T)_n$, as a synthetic helical DNA, does not possess any specific promoter sites as are present in natural nucleic acids. Hence, the high general level of binding displayed by RNA polymerase toward polyanions, and the initiation reactions that occur with high frequency at ends (Vogt, 1969) and nicks (Dausse et al., 1972) (of which there are probably many in $d(A\text{-}T)_n$) means that a very large proportion of the RNA polymerase will be actively engaged in transcription of the $d(A\text{-}T)_n$ effectively causing saturation. Under the assay conditions of 0.2M potassium ion concentration, as discussed above, there is less inhibition by a single stranded nucleotide anyway.

However, if the kinetics are studied with a natural double stranded DNA template, initiation only occurs at the small number of promoter sites in the nucleic acid (assuming its integrity as far as nicks and ends are concerned). Although it will still bind RNA polymerase non-specifically, such "closed" complexes are susceptible to displacement by other polyanions, in this case $(z^8A, A)_n$, since holoenzyme-double stranded DNA complexes have only low binding constants (de Haseth et al., 1978). Because of the limited number of initiation sites available, there should be a noted dependence of the rate of reaction on increasing DNA concentration, due to the large amount of free RNA polymerase (free with respect to DNA binding) present.

The results of kinetic experiments with calf thymus DNA as template in the presence of $(z^8A, A)_n$ are shown in Figure 3.12. The Lineweaver-Burk plot shows non-competitive inhibition of calf thymus DNA transcription; from the normal interpretation of such plots, it would appear that $(z^8A, A)_n$ inhibits the enzyme by binding at a site distinct from the template binding site. This was unexpected, but an appreciation of the complex nature of the binding phenomena involved in template binding, initiation and elongation shows that true competitive inhibition does

Figure 3.12

Lineweaver-Burk plot for inhibition of calf thymus DNA transcription by $(z^8A, A)_n$. Incubations (37° C) were performed in 0.04 M bicine, pH 8.0, 0.2 M KCl, 10 mM $MgCl_2$ containing calf thymus DNA (0.1-1.0 mg/ml), GTP, UTP, CTP and [^{14}C]-ATP all at 0.5 mM and enzyme at 20 μg/ml (0.1 ml total volume).

For inhibition, $(z^8A, A)_n$ (z^8Ado content = 13.4%) was added (28 μM) prior to addition of enzyme (×—×—×).

1/V (min.nmole[14C]-ATP-1. × 10-2)
1.0
0.5
0
5
10
1/S (ml.µg-1)

not exist in this system because of the largely irreversible nature (on the timescale involved here) of template-RNA polymerase binding (Wood and Berg, 1964; Fox et al., 1965). The determination of enzymic activity uses an assay based on incorporation of mononucleotides into polynucleotides (i.e. basically the elongation step is being monitored (Bremer, 1967))where very stable binary complexes are present which are not prone to displacement (and hence not susceptible to direct competitive inhibition). As regards competition at sites other than the template binding site, the active site of RNA polymerase is very ill-defined, but seems to contain,at the minimum, a template site, a catalytic site comprising initiation and product terminus sites, as well as a larger product site where nascent RNA is located (Krakow et al., 1976). Hence, any interpretation of the non-competitive data is fraught with uncertainty, but interestingly the elongation inhibitor, streptolydigin, also displays non-competitive inhibition of the enzyme when the nucleoside triphosphate concentration is varied (Cassani et al., 1971), so that the present result is not entirely without precedent. A consideration of the published data show two possibilities exist such that the rate of RNA transcription could be lowered by RNA without affecting the affinity of the enzyme for the natural template.

(a) Kumar and Krakow (1975) have discovered that in the ternary complex, a length of RNA (10-12 residues) is protected by the enzyme from the effects of ribonuclease. There is no evidence, however, for the existence of DNA-RNA hybrids in the active site, so it is concluded that distal regions of the RNA product site diverge from the template site such that re-association of the DNA duplex is favoured. Binding of the $(z^{8}A, A)_{n}$ in such regions of the RNA product site could certainly be expected to slow the efficiency of transcription.

(b) More recently, Rohrer and Zillig (1977) have shown that in the actively transcribing complex of the elongation phase, the non-coding strand of the DNA template is in a much more exposed position with

regard to single stranded specific nuclease digestion, suggesting that the two strands of the DNA are separated at two distinct sites. Previous studies on the susceptibility of stable core enzyme non-transcribing complexes to displacement by polynucleotides (Hinkle and Chamberlin, 1972) have suggested direct displacement can occur, probably via binding to a second nucleic acid binding site. Hence, it is possible that competition with the non-transcribed strand at this second (non-catalytic) binding site by $(z^8A, A)_n$ could also account for the observed kinetics.

The foregoing discussion shows that investigation of the binding site of $(z^8A, A)_n$ under conditions where ternary complex formation has occurred with double stranded DNA is not applicable to the binding site adopted in a simple binary complex of $(z^8A, A)_n$ and enzyme. That this site is in fact the template site of the enzyme could be inferred from the "order of addition" experiments reported above and the fact that the polynucleotide is a template. Further support for this notion comes from experiments on the photolytic inactivation of the enzyme under different conditions.

(ii) Photolysis of binary complexes of $(z^8A, A)_n$ and RNA polymerase

In Figure 3.13, the activity of enzyme after 30 seconds photolysis at different concentrations of $(z^8A, A)_n$ is shown. The points lie on a straight line in the semi-log plot, denoting the first order nature of the photolysis reaction. After rather more extended periods of photolysis, higher order processes appear to take place as the azide has presumably all been photolysed at this stage. Rapid inactivation of enzyme occurs at low concentrations of inhibitor under the optimum salt conditions for binary complex formation used (Figure 3.14).

The results of protection experiments are also shown in Figure 3.14. Good protection is afforded by the helical template, $d(A\text{-}T)_n$. On addition of adenosine at approximately equivalent optical density, no protective effect was seen, showing that the protection observed is not due to preferential absorbance of the light (i.e. a filtering effect).

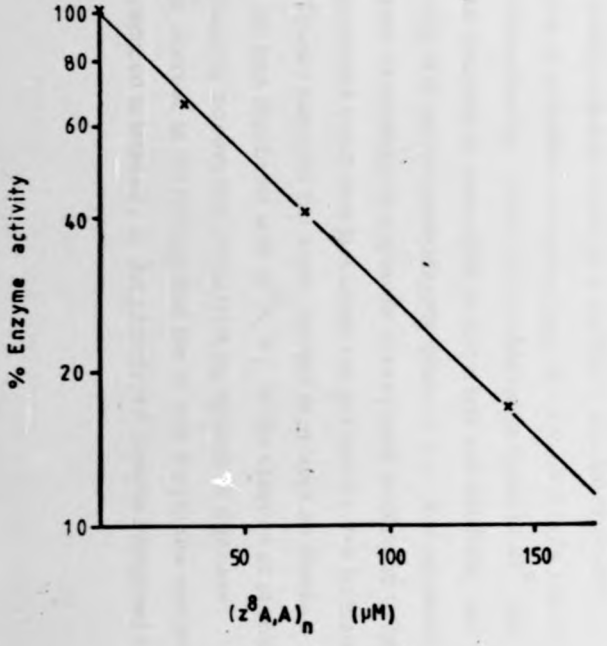
Figure 3.13

Photolytic inactivation of RNA polymerase in the presence of $(z^8A, A)_n$. Incubation prior to photolysis for 10 minutes at 37° was performed in 0.05 M potassium buffer containing 4 mM Mn^{2+}. The extent of enzyme activity was monitored after 30 seconds photolysis (filter used was pyrex/soda glass combination of Figure 2.17) and plotted on a logarithmic scale versus concentration of $(z^8A, A)_n$ (z^8Ado content = 13.4%). Enzyme concentration was 0.2 mg/ml.

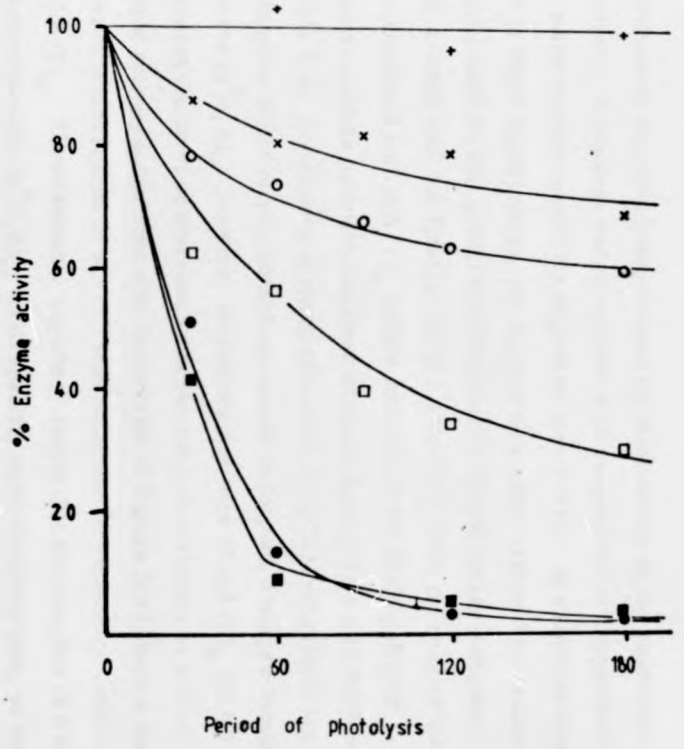
Figure 3.14

Effect of differing treatments on the photolytic inactivation of RNA polymerase. Enzyme was added to the following incubation mixtures (at a final concentration of 0.2 mg/ml) and held at 37° for ten minutes prior to photolysis for differing periods. 0.05 M potassium buffer + A_n (+ — +), buffer + $(z^8A, A)_n$ (28 μM) (□ – □ – □), buffer + $(z^8A, A)_n$ (70 μM) (■ – ■ – ■), buffer + $(z^8A, A)_n$ (70 μM) + adenosine (0.5 A_{260} units) (● – ● – ●), buffer + $(z^8A, A)_n$ (28 μM) + $d(A\text{-}T)_n$ (30 μM) (O – O – O), buffer + $(z^8A, A)_n$ (28 μM) + $d(A\text{-}T)_n$ (90 μM) (X – X – X). $(z^8A, A)_n$ sample contained 13.4% z^8Ado.

3.13


100
80
60
40
20
10
% Enzyme activity
50
100
150
$(z^8A,A)_n$ (μM)

3.14


100
80
60
40
20
% Enzyme activity
0
60
120
180
Period of photolysis

Photolysis of enzyme alone produces no inactivation on the timescale involved. Incubation and photolysis with magnesium or manganese ion made no difference to the degree of inactivation. It should be noted that in these experiments, the enzyme was added last to the mixture of templates so that preferential formation of one binary complex over another was not likely to occur. However, when the enzyme was pre-incubated with $d(A\text{-}T)_n$ before addition of the azido polymer, almost complete protection against enzymic inactivation was achieved (Table 3.1). Conditions of incubation were at 0.05 M potassium ion so that tightly bound binary complexes would be formed. When pre-incubated with the $(z^8A, A)_n$; however, increasing amounts of $d(A\text{-}T)_n$ did not decrease the level of inactivation achieved; that there was a base level of activity correlates with the results of Figure 3.10 where a base level of transcription of d(A-T) still occurred on secondary addition of $d(A\text{-}T)_n$. These results together imply that the complex of RNA polymerase with $(z^8A, A)_n$, while specific at the template site, is not of such a tightly bound nature as that with $d(A\text{-}T)_n$.

(iii) Analysis of products of photolysis

In order to achieve conditions where a large proportion of the enzyme molecules would be bound to template, analysis of the products of photolysis was made on a series of incubations with differing ratios of polymer to enzyme, i.e. from 0.65:1 (w/w) up to 7.8:1 (w/w) respectively. After 2.5 minutes photolysis at 22°, the products were isolated as described in Materials and Methods, and run out on 5-15% SDS polyacrylamide gels (Plate 3.1). It is apparent that in all cases of photolysis a number of very high molecular weight bands are generated which only just enter the gel (tracks 2-8 inclusive, arrowed). Track 9 contains the same amount of protein as tracks 2 and 3 (8 µg), but was incubated with $(z^8A, A)_n$ in the absence of photolysis, thus demonstrating no covalent cross-linking occurs in this case. Of particular interest is the finding that the β' and β subunits are both drastically reduced in intensity in the photolysed sample compared to

Table 3.1

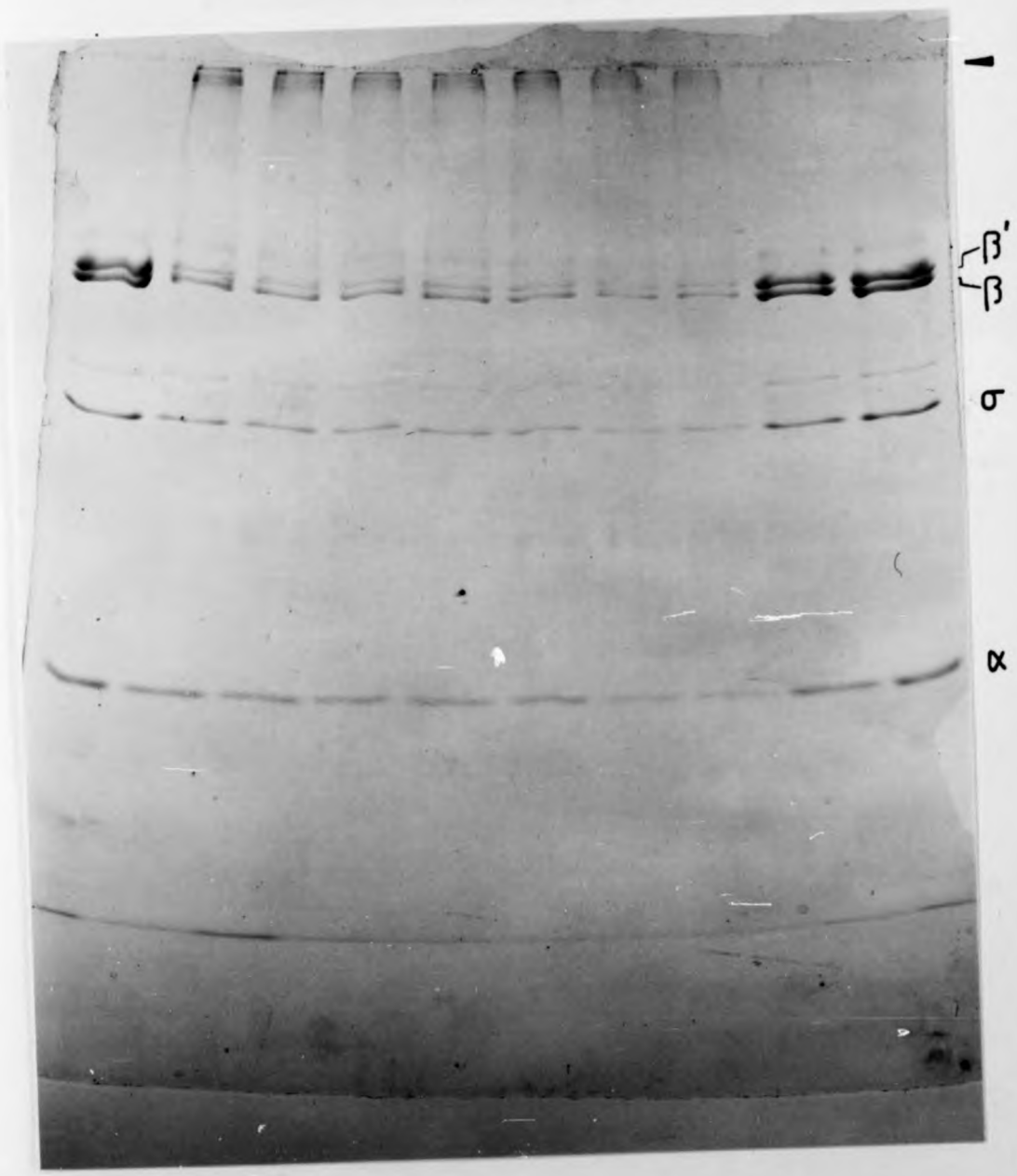
Effect of "order of addition" on protection of E. coli RNA polymerase against photoinactivation by $(z^8A, A)_n$

First addition		Second addition		Enzyme activity
Polymer	Concentration (μM)	Polymer	Concentration (μM)	%
$(z^8A, A)_n$	66.6	–	–	5.4
$d(A\text{-}T)_n$	82.5	$(z^8A, A)_n$	66.6	79.2
$d(A\text{-}T)_n$	165	$(z^8A, A)_n$	66.6	94.5
$(z^8A, A)_n$	66.6	$d(A\text{-}T)_n$	82.5	42.9
$(z^8A, A)_n$	66.6	$d(A\text{-}T)_n$	165	51.8
Adenosine	130	$(z^8A, A)_n$	66.6	4.3

Incubations prior to photolysis were performed in 0.04 M bicine, pH 8.0, 0.05 M KCl, 0.01 M $MgCl_2$ for 15 minutes at room temperature. Polynucleotide, at the desired concentration, and enzyme (20 μg) were mixed together initially, and after 15 minutes the second addition was made and photolysis for 6 minutes immediately commenced. Enzyme aliquots were withdrawn and assayed for activity as described in Section 3.2.4 in 0.2 M KCl-containing buffer.

Plate 3.1

5-15% gradient SDS gel electrophoresis of *E. coli* RNA polymerase after photoinactivation with $(z^8A, A)_n$. Samples were incubated and photoinactivated (as described in Section 3.2.5b) in 0.1 ml of buffer, 2.5 minutes per sample at 22°. They were subsequently prepared for electrophoresis as described in Section 3.2.6. Tracks 1 and 10 contained 10 μg of native RNA polymerase as markers. Track 9 contained 8 μg of enzyme photolysed in the absence of any substrate. Tracks 2 and 3 both contained 8 μg of enzyme and 0.143 mM and 0.286 mM respectively of $(z^8A, A)_n$ (13.4% z^8Ado content). Tracks 4, 5 and 6 all contained 4 μg of enzyme and 0.143 mM, 0.286 mM and 0.429 mM $(z^8A, A)_n$ respectively. Tracks 7 and 8 contained 2 μg of enzyme and $(z^8A, A)_n$ at 0.286 and 0.429 mM respectively. On a weight to weight basis, the ratios of polymer to enzyme ranged from 0.65:1 to 7.8:1. β', β, σ and α represent the four major subunits; the arrow denotes the high molecular weight bands generated.


1
2
3
4
5
6
7
8
9
10
β'
β
σ
α

the unphotolysed, whilst the σ and α subunits remain virtually unaffected. In fact, the β' subunit is, if anything, slightly more reduced in intensity than the β (tracks 2, 3 and 9 for comparison). An important conclusion from these observations is the specific nature of the cross-linking reaction; if a high degree of non-specific or "pseudo" photoaffinity labelling were occurring, then the σ and α subunits would be drastically reduced in intensity also. After this work was completed, Okada et al. (1978) showed substantially the same results in photolabelling studies, using Brd Urd-substituted DNA as a template for the enzyme. With increasing periods of irradiation at 305 nm, they found a progressive decrease in the intensity of the β and β' subunits under salt conditions where specific complex formation was taking place. As the ratio of template to enzyme was decreased below 10:1 (w/w), they found a progressive loss of specificity and all subunits were found to disappear from the gel. Under the conditions used in the present investigation, highly specific cross-linking was still observed, even at ratios less than 1:1 (w/w).

It was of interest to look at the gel patterns that would be obtained using a variety of protective conditions, as performed in the kinetic experiments. The results (Plate 3.2) show that significant protection by $d(A\text{-}T)_n$ as evidenced in the kinetic experiments, is also manifest from the gel patterns. All tracks were loaded with 8 μg of protein and hence are directly comparable. Tracks 3 and 4 show that no cross-linking is produced when the enzyme is photolysed in the presence of either $d(A\text{-}T)_n$ or A_n respectively, while the normal high level of cross-linking is produced in the presence of $(z^8A, A)_n$ (track 7). In track 8, pre-photolysed azido polymer shows no cross-linking on photolysis in the presence of the enzyme, as is required for a photo affinity label. Pre-incubation of enzyme for 15 minutes with $d(A\text{-}T)_n$, followed by 5 minutes incubation with $(z^8A, A)_n$ reduced the extent of cross-linking to a marked extent, and as seen in track 3, the intensity of the β and β' bands is almost as intense as in control tracks. The reverse

Plate 3.2

Effect of protective agents on the patterns produced on SDS gel electrophoresis after photolysis of RNA polymerase with $(z^8A, A)_n$. Buffer conditions of incubation and photolysis were the same as in Plate 3.1. All tracks contain 8 μg of enzyme prepared for electrophoresis as in Section 3.2.6.

Track 1 contained enzyme incubated alone, and not photolysed.

Track 2 contained enzyme incubated for 15 minutes with $(z^8A, A)_n$ (0.0715 mM), but not photolysed.

Track 3 enzyme was incubated with $d(A\text{-}T)_n$ (0.147 mM) for 15 minutes at 37° C prior to addition of $(z^8A, A)_n$ (0.0715 mM) and immediate photolysis.

Track 4 as for track 3, but incubation régime reversed.

Track 5 contained enzyme incubated with A_n (0.208 mM) for 15 minutes prior to photolysis.

Track 6 contained enzyme incubated with $d(A\text{-}T)_n$ (0.147 mM) for 15 minutes prior to photolysis.

Track 7 contained enzyme incubated with $(z^8A, A)_n$ (0.0715 mM) for 15 minutes prior to photolysis.

Track 8 contained enzyme incubated with pre-photolysed $(z^8A, A)_n$ (0.0715 mM) and then photolysed further after 15 minutes.

β', β, σ and α represent the four major subunits; the arrow indicates the high molecular weight bands generated.


1
2
3
4
5
6
7
8
β'
β
σ
α

incubation, i.e. $(z^8A, A)_n$ first, followed by $d(A\text{-}T)_n$ shows a rather similar pattern (track 4) in terms of reduced cross-linking although this is not so marked as in track 3 (see high molecular weight bands).

Frischauf and Scheit (1973) used the photolabile deoxypolynucleotide, $d(s^4T)_n$, to inactivate RNA polymerase. They found, however, that inactivation was not competed very effectively by $d(A\text{-}T)_n$, even at a 10-fold molar excess, in contrast to the results reported here (Table 3.1). On SDS polyacrylamide gel electrophoresis of a sample inactivated with $[^{32}P]$-labelled polymer, the peaks of radioactivity were associated with high molecular weight bands and also a small amount with the region of the β' subunit (which was poorly resolved from the β subunit). We performed similar experiments with a $[^3H]$-labelled sample of $(z^8A, A)_n$ and gained very similar results (Figure 3.15). No counts were associated with the σ and α subunits. Since the counts obtained in the region of β, β' were on the high molecular weight side of the band (β' = MW 165,000; β = MW 155,000), Frischauf and Scheit assumed that primary cross-linking to β' occurred. In the present experiments we decided to excise the high molecular weight bands, extract the products and digest the nucleic acid with ribonuclease to regenerate the subunits (since any counts found in the β', β region could be due to degradation products and not from genuine cross-linking).

When the products of this treatment were run out on gels and stained for protein (Plate 3.3) it was clear that only the β' and β subunits had been involved in the binding of template $(z^8A, A)_n$ (tracks 5, 6 and 7; see track 2 for comparison with native enzyme). Compared to the work of Frischauf and Scheit (1973) and Okada et al. (1978), this is the first direct demonstration of the identity of the subunits cross-linked to template after photolysis of binary complexes, and leads to the conclusion that the primary binding site (at least before transcription is initiated) for nucleic acids lies across the β and β' subunits. It would certainly be interesting to look at the pattern generated by photolysis of the ternary complex of enzyme/$(z^8A, A)_n$/nascent U_n (which could be isolated by gel filtration) in order to compare the results. This would give an insight into whether conformational changes occur during initiation and/or elongation stages.

The subunit topography of RNA polymerase has been the subject of

Figure 3.15

Distribution of radioactivity in SDS-phosphate gels after electrophoresis of $(z^8A, A)_n$ photoinactivated RNA polymerase. Samples of enzyme (80 μg) were preincubated with $([^3H)\text{-}A, z^8A)_n$ (84.5 μM; sp. Act = 6230 cpm/nmole) in 0.05 M potassium ion buffer (0.1 ml) containing 10mM Mg^{2+}, for 10 minutes at 37° C. Photolysis for four minutes was followed by preparation of samples for gel electrophoresis by addition of 2-mercaptoethanol (0.001 ml), SDS (ca. 1 mg) and boiling for 1 minute. Glycerine (0.025 ml) was added, together with bromophenol blue marker dye, and 0.04 ml samples were run on 5% SDS-phosphate gels as described in Section 3.2.2. The gels were sliced into approximately 2mm thick pieces and depolymerised by heating with 0.3 ml of 30% H_2O_2 at 50° C for 2-3 days. Radioactivity was determined in 10 ml of aquasol.

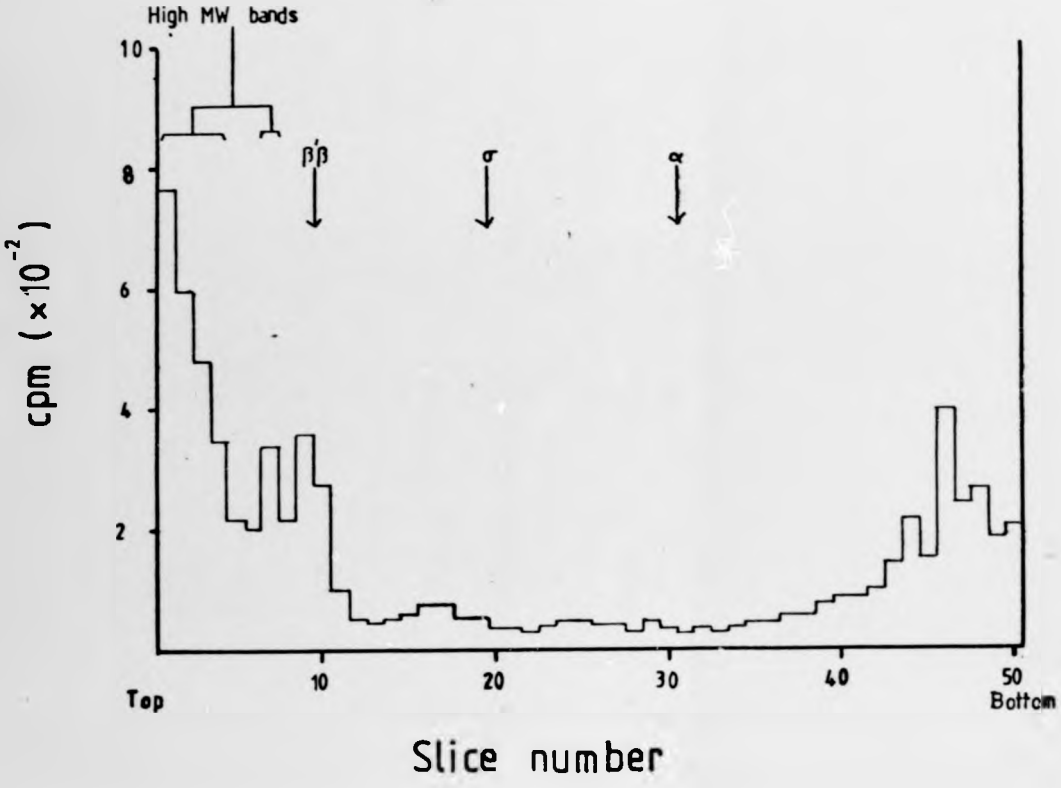
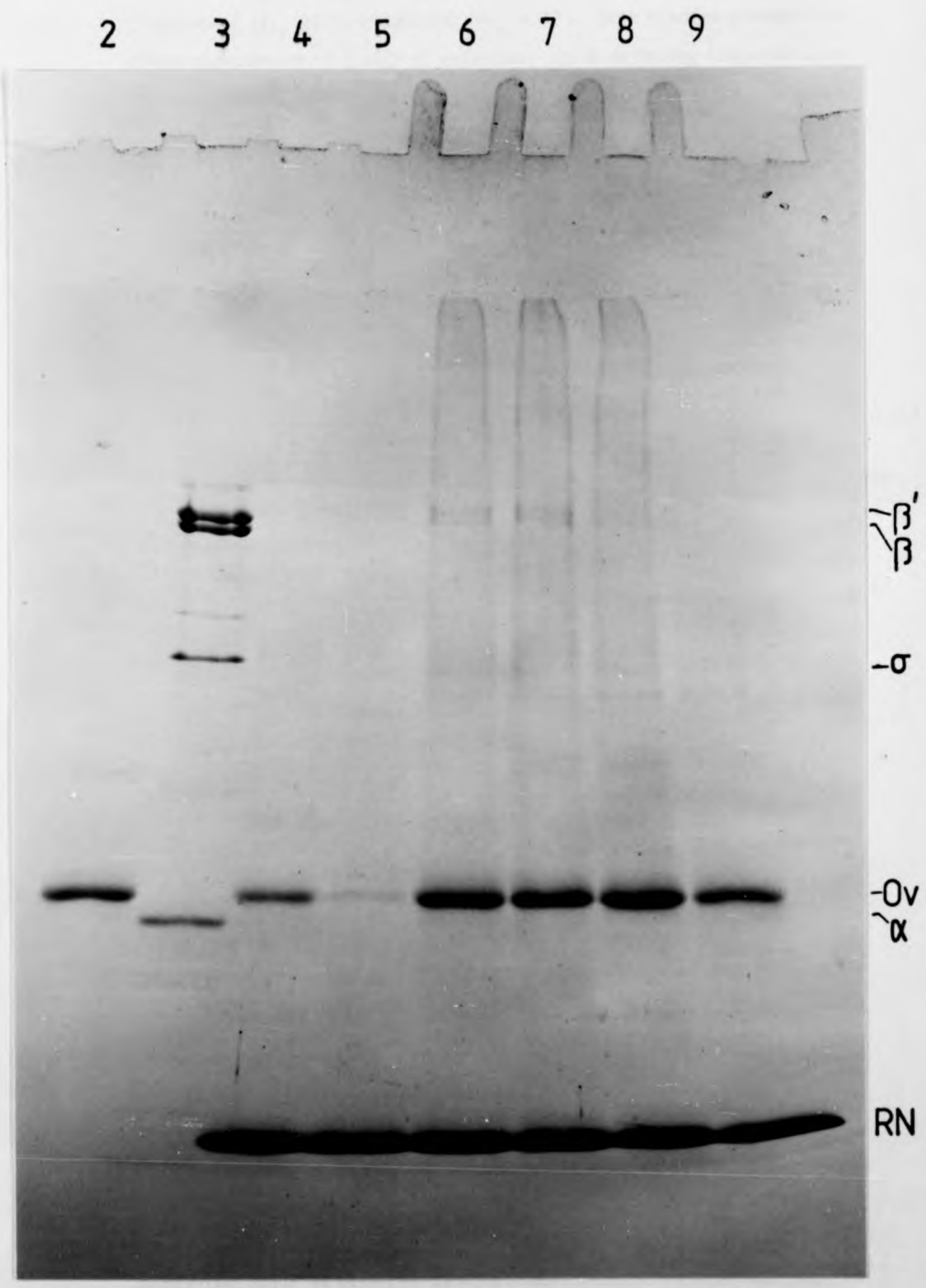

High MW bands
β'β
σ
α
cpm (×10⁻²)
10
8
6
4
2
Top
10
20
30
40
50
Bottom
Slice number

Plate 3.3

Demonstration, by SDS gel electrophoresis, of the identity of RNA polymerase template binding subunits. The conditions of the experiment are fully described in Section 3.2.6. Tracks 5-8 inclusive were loaded with hydrolysed high molecular weight material extracted from a gel similar to that in Plate 3.1. Track 5 was protein from a photoinactivation performed in 0.05 M potassium ion buffer (0.2 ml) with enzyme (16 μg) pre-incubated with 0.143 mM $(z^{8}A, A)_n$ (this material was, however, mostly lost prior to separation on the present gel). Tracks 6-8 are similar, but with different relative concentrations of enzyme and photolabile polynucleotide, i.e. enzyme (16 μg) and $(z^{8}A, A)_n$ at 0.286 mM (track 6) and 0.574 mM (track 7), or enzyme (10 μg) and $(z^{8}A, A)_n$ at 0.429 mM (track 8). Track 2 contains ovalbumin (12.5 μg), track 3, native RNA polymerase (10 μg); tracks 4 and 9 control ovalbumin and ribonuclease taken through the same precipitation processes as the cross-linked RNA polymerase subunits.


2
3
4
5
6
7
8
9
β'
β
σ
Ov
α
RN

two non-specific cross-linking approaches using bis-imidates (Coggins et al., 1977; Hillel and Wu, 1977). Both studies showed the close association of β and β' subunits, while differing in the details regarding the disposition of α and σ subunits. It is clear from the present results that the α subunits are not in any way near the template binding site, which spans the two large subunits. No information with regard to σ can be derived from this study since binding of holoenzyme ($\beta'\beta\alpha_2\sigma$) to single stranded polynucleotides lands to displacement of σ subunit (Krakow and von der Helm, 1971).

While the preparation of this thesis was underway, a report on the cross-linking of T7 DNA to E. coli RNA polymerase appeared (Hillel and Wu, 1978). This is a very detailed study on the nature of the binding of DNA in both "non-specific" as studied here and "specific" (involving promoter sites) modes. No synthetic photoaffinity probe was used; rather the complexes were irradiated with damaging 254 nm UV light, which in the absence of template destroyed up to 50% enzymic activity. Apart from this caveat (which, it was claimed, did not affect the integrity of binding interactions) the results for non-specific binding are in accord with those reported here. As well as β and β' subunits, σ was also linked to the template (in Plate 3.3, faint traces of σ can be seen in tracks 5, 6 and 7). In contrast to this, in specific "open" complexes as well as ternary initiation complexes, only the β and σ subunits were cross-linked. This was a rather surprising result in view of the fact that β' is the only isolated subunit to have any affinity for nucleic acids. On the other hand, β does contain the nucleoside triphosphate sites, so must be considered to be part of the synthetic centre in initiation and elongation phases. It was suggested that β' may be used for rapid non-specific attachment to DNA, thus facilitating the search for promoter regions by attachment via a "ring closure" type mechanism to separate regions of DNA. When a promoter site was located (the function of the σ subunit) the β' would move away from the template, so allowing the β subunit to perform its catalytic function. These are very interesting results and would be well complemented by a study of the ternary complex with a single stranded template such as described here, in order to assess whether single strands can cause the same types of conformational change.

In conclusion, it has been demonstrated that the synthetic azido-containing polynucleotide, $(z^8A, A)_n$, can be employed to give useful structural information in photoaffinity labelling experiments. Specifically, it was shown that the β' and β subunits of RNA polymerase contain the template binding site of binary complexes. The correctness of the association was shown by competition experiments and the non-random cross-linking achieved (non-specific binding should have cross-linked all subunits). These synthetic polynucleotides should be able to provide structural information in other systems also, e.g. by acting as mRNA in in vitro protein synthesis to probe ribosomal interactions, or in studies involving antibodies directed against both single stranded and double stranded forms. In connection with this latter point, samples of $(z^8I, I)_n.C_n$ prepared here are being investigated for recognition by anti-$I_n.C_n$ antibodies (Dr. M. Leng, CNRS, Orléans, France). This could be important for studies related to interferon inhibition (see Chapter Four) as well as a possible probe of binding regions in antibodies.

Note added prior to submission

Dr. Leng has found that the $(z^8I, I)_n.C_n$ complex reacts well with antibodies directed against double stranded RNA, providing a link with the demonstration of its interferon-inducing capability (see Chapter Four).

CHAPTER FOUR

4.1 Introduction

4.1.1 Induction of interferon by polynucleotides

Since the discovery of the antiviral substance interferon in 1957 (Isaacs and Lindenmann, 1957), there have been innumerable studies aimed at elucidation of both its mechanism of induction and its mode of action. Interest has been aroused because of two novel and important features displayed by the interferon system. Firstly, it is one of the few inducible eukaryotic gene systems known, and as such is likely to provide much basic information regarding control and regulation in eukaryotic gene expression. Secondly, the wide spectrum of viruses against which interferon displays its inhibitory action (Merigan, 1964) makes it a valuable tool, not only in prophylaxis but in basic research aimed at understanding the molecular basis of viral interference. It will be shown that these two important features are not unconnected in that an appreciation of factors that lead to high yields of interferon is likely to be of some clinical importance.

Not only is interferon active against a wide variety of viruses, it can also be induced *in vivo* and *in vitro* by an equally broad range (Ho, 1973). Isaacs (1963) first suggested that the induction of interferon was a response to foreign nucleic acids, but several efforts to verify this met with inconclusive results. Eventual proof came when it was shown that both natural and synthetic double stranded RNA were efficient inducers of interferon both *in vivo* and in cells in culture (Lampson *et al.*, 1967; Field *et al.*, 1967, 1968). In particular, the synthetic inducer, $I_n.C_n$, was found to be the best inducer of those tested, and this species has become one of the standard investigative reagents in interferon research.

A number of studies has , however, shown that some major differences between the mechanisms of induction by viruses and by double stranded RNA *in vitro* exist:

(a) The kinetics of response of interferon production are markedly different. Thus, treatment with $I_n.C_n$ leads to peak titres at about 4 hours post-induction with a subsequent rapid decrease in levels. Induction with NDV on the other hand leads to a much later peak response and one which continues for a longer period (Mozes and Vilcek, 1975).

(b) Treatment of chick or human cells with the alkaloid camptothecin inhibits interferon induction by NDV or Sindbis virus, but has no effect on $I_n.C_n$ induction (Atherton and Burke, 1975, 1978).

(c) Pre-irradiation of cells with moderate doses of UV leads to greatly increased levels of interferon when induced by $I_n.C_n$, but a marked fall is apparent after NDV induction. The kinetics of the $I_n.C_n$ induced interferon response became similar to those for normal viral induction (see (a) above) (Mozes and Vilcek, 1974, 1975).

(d) Early studies on the effect of metabolic inhibitors such as actinomycin D and cycloheximide revealed the relatively greater resistance of $I_n.C_n$ induction to such treatments (e.g. Long and Burke, 1971). A hypothesis that virus induction was accompanied by de novo interferon synthesis, whilst double stranded RNA could release pre-formed "pools" of interferon was advanced (Youngner and Hallum, 1968). Other workers, however, showed conclusively that de novo synthesis was required in both cases (Finkelstein et al., 1968; Tan et al., 1970) but that the response of the polynucleotide induced system was of a rather complex nature.

This latter point has been the subject of much subsequent investigation since it bears directly on the nature of the control of interferon induction as mediated by exogenously added double stranded RNA. Of particular importance was the observation that much higher levels of interferon than normally expected from $I_n.C_n$ treatment could be produced by a suitable regime of treatment with actinomycin D and cycloheximide, a phenomenon known as "superinduction" (Vilcek and Ng, 1971). Thus, cells induced with $I_n.C_n$ in the presence of cycloheximide produce a burst of interferon when the cycloheximide

is washed out of the system after 4 hours. If the experiment is repeated but actinomycin D is added for one hour before the cycloheximide is washed out, not only is the burst of interferon still observed but it is also larger and more protracted, the kinetics of production resembling those of viral induction. These results were interpreted as evidence that a post-transcriptional control on interferon synthesis was operative. In an early formulation, Ng and Vilcek (1972) proposed that a repressor protein, produced by constitutive synthesis from a repressor gene, bound to interferon mRNA and inhibited its translation. This mRNA complex exerted negative control on the interferon gene itself. Induction was presumed to occur by preferential binding of the repressor protein to the double stranded RNA, allowing translation of the interferon mRNA and hence further transcription of the interferon gene. Under these circumstances, the effects of the metabolic inhibitor are readily interpretable, since during the period of cycloheximide treatment, the synthesis of repressor protein would be inhibited, and levels of interferon mRNA would build up. Addition of actinomycin D would halt transcription of the genes. Thus, on washing out the reversible inhibitor cycloheximide, translation of the interferon mRNA could proceed, whilst the block on transcription would prohibit any synthesis of repressor mRNA for translation into repressor protein. Hence, interferon production would be higher and last for longer.

A number of points are raised in this hypothesis, some of which have become directly amenable to experimental trial. Of most importance in this respect is the fact that interferon mRNA can now be isolated and transcribed, both in cell-free systems (Pestka et al., 1975) and in heterologous cell systems (De Maeyer-Guignard et al., 1972; Reynolds et al., 1975). These studies (and others) have shown conclusively that no interferon mRNA exists in uninduced cells, so that induction as seen by Ng and Vilcek (1972) is untenable and must involve de novo transcription of the interferon gene. Further, it is by no means clear

how the double stranded RNA induces or derepresses the interferon gene, but recent results (Marcus and Sekellick, 1977) disclose it to be very sensitive to the presence of single molecules of double strand, clearly a process not involving binding to a large number of rapidly turning-over repressor molecules (see further discussion later). Using the Xenopus oocyte translation system, Cavalieri et al. (1977a) have directly quantitated the levels of interferon mRNA in normally induced cells and those superinduced with cycloheximide alone or by a combination of cycloheximide and actinomycin D. It is found that under the superinduction conditions, larger quantities of interferon mRNA are produced and that this has a longer half-life than the mRNA synthesised by normal $I_n.C_n$ induction. Rather similar results have been obtained by Seghal et al. (1977). The post-transcriptional repressor hypothesis remains valid, but the proposed repressor protein may well act by increasing the rate of inactivation of interferon mRNA, as previously proposed (Vilcek and Havell, 1973). Under these circumstances, there is no requirement for a repressor protein to be constitutively synthesised since in uninduced cells there is no mRNA on which to act. It seems that the scheme shown in Figure 4.1 is the most likely explanation of control based on present data. Both the interferon gene and the repressor gene are inducible; interferon itself exerts positive control over the induction of the repressor protein, thus explaining the rapid shut-down in interferon synthesis.

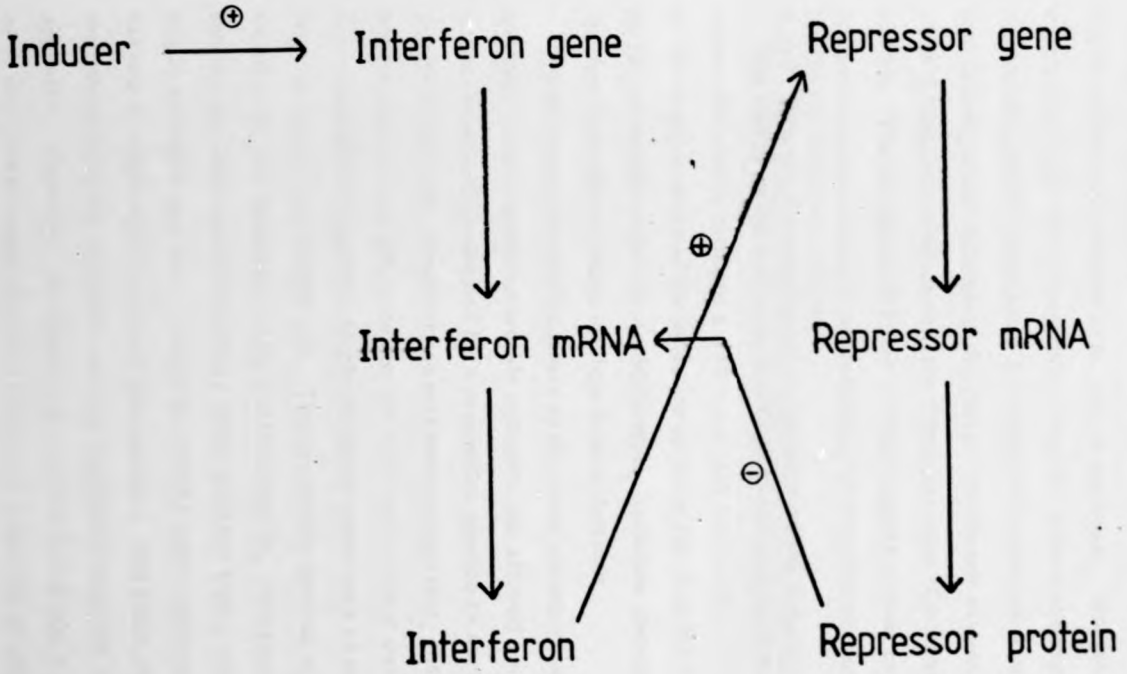
The effect of UV irradiation on the induction by $I_n.C_n$ seems likely to involve preferential destruction of the repressor gene system and, in support of this, UV treatment of a superinduced system shows the normal dose-response curve of inactivation as seen for virus induction (Mozes and Vilcek, 1974). Thus, while a plausible model has arisen for double stranded RNA control of interferon synthesis (the molecular details of induction itself remaining unknown), the same is certainly not true for viral control. It does, however, appear that destruction of the repressor system leads to a form of

Figure 4.1

Hypothetical scheme for control of interferon synthesis

⊕ denotes an activation (or derepression) process

⊖ denotes an inhibitory (or degradative)process


Inducer
⊕
Interferon gene
Repressor gene
⊕
Interferon mRNA
Repressor mRNA
⊖
Interferon
Repressor protein

induction by ds RNA akin to that of viruses. The interferons induced by either agent are antigenically indistinct from one another (Havell et al., 1975), and so it seems likely that both are produced from the same gene system, but under rather different mechanisms of control. The basis for this difference is, as yet, unknown. Recent results with human cells have shown other complex control mechanisms exist at the genetic level since leucocyte cells produce a mixture of both leucocyte and fibroblast interferon (which are antigenically distinct) while fibroblasts produce only the fibroblast type (Cavalieri et al., 1977b). The resolution of these various aspects almost certainly requires a more thorough understanding of the molecular mechanism behind the induction process.

4.1.2 Structural requirements for polynucleotide inducers

The ability of the synthetic double stranded polynucleotides to induce interferon initiated a more rational approach to investigations of the requirements of the induction process, for such RNA species are highly amenable to chemical modification, and allow detailed studies on structure-function relationships to be undertaken.

A wide variety of modifications to the basic structure of the synthetic homopolyribonucleotide inducers has allowed a number of strict criteria to be defined for a particular species to serve as an interferon inducer. However, as will become apparent, these criteria are necessary requirements but not sufficient on their own.

(a) All inducers possess double stranded structures of the normal Watson-Crick base paired type. Triple strands such as $A_n.2U_n$ were found to be non-inducers (Colby and Chamberlin, 1969) and this finding has been repeated by many other workers with a variety of triple stranded species. Ambiguous results were obtained with a variety of single stranded homoribopolymers, and some reports of interferon inducing capability at very high concentrations of polymer appeared. Recently, De Clercq's group have found that a preparation of I_n was an extremely efficient inducer in a variety of cell lines, with titres approaching that of $I_n.C_n$ in some cases (Thang et al., 1977; De Clercq et al., 1978a). However, it is well known that preparations of I_n have the ability to form multi-stranded structures (Thiele and Guschlbauer, 1973) and a close examination of the inducing species

showed them to react strongly with antibodies directed against ds RNA, but not with antibodies against ss RNA (Stollar et al., 1978).
(b) All inducers possess adequate T_m's and resistance to nuclease under physiological conditions. These two properties appear to be a statement on the overall stability of the secondary structure of the double stranded inducer and are thus not mutually exclusive. A complex with a high T_m will necessarily be more resistant to nucleases due to the smaller amount of "breathing" of the base pairs at physiological temperatures (this argument necessarily ignores double strand specific nucleases). Experimentally, it is found that complexes with T_m above 60^o C are the most efficient inducers, those with T_m between 40^o and 50^o are of intermediate efficiency, and those with T_m below 40^o are non-inducers (De Clercq, 1974). However, possession of both properties does not mean that a given complex will induce interferon, e.g. the complexes $(c^7A)_n.U_n$ (Torrence and Witkop, 1975) and $X_n.A_n$ (Torrence et al., 1977) both possessed high T_m's as well as the other criteria noted here, but neither were active as interferon inducers. On the other hand, inducers with very high values of T_m, e.g. $G_n.C_n$ (with $T_m > 100^o$ C) are no more efficient inducers than inducers with lower T_m values (e.g. $I_n.C_n$, T_m 63^o C)(Novokhatsky et al., 1975).
(c) All inducers possess a minimum threshold molecular size, below which inducing capability disappears. Various studies have concentrated on reduction in the size of one strand of the $I_n.C_n$ complex in relation to the other. The general conclusion appears to be that effective interferon inducers are between 5S and 10S in size, and that the I_n strand is more sensitive to changes in molecular size than the C_n strand (Pitha and Hutchinson, 1977).

A recent study has questioned the validity of high molecular size as a general statement of requirement for interferon induction. Using a series of $I_n.C_n$ analogues with differing degrees of 2'-O-methylation in each strand, it was found that while high molecular size was important for initial binding to the putative ds RNA receptor, subsequent recognition of helical segments of only 6-12 base pairs

could trigger interferon production (Greene et al., 1978) (see below for further discussion of these results.

(d) All inducers possess ribose sugar containing 2'-OH in both strands. Early studies showed that neither ds DNA nor ds DNA/RNA hybrids were able to induce interferon (Vilcek et al., 1968; Colby and Chamberlin, 1969). Many polymers have been tested in which the 2'-OH of one strand has been completely replaced by a variety of groups such as alkyl, halogen and O-acyl. None of these were shown to possess interferon inducing properties, even though they all demonstrated high nuclease resistance, and it was long assumed, therefore, that the 2'-OH was an absolute requirement (see Torrence and De Clercq, 1977, for review). Partial replacement of the 2'-OH of one strand has given conflicting results. Thus, Steward et al. (1972) introduced 2'-O-acetyl groups into the A_n, I_n and C_n ribopolymers. Substitution at levels greater than 50% led to inactivity of complexes as inducers, whereas low levels of substitution (6%) led to complexes with an activity approaching that of the parent complexes. On the other hand, partial 2'-O-methylation of I_n and and C_n led to polymers that possessed a high inducing ability, even at very high degrees of substitution, e.g. 80% substitution by 2'-OMe in the I_n strand of $I_n.C_n$ produced virtually no reduction in the anti-viral effect (Merigan and Rottman, 1974). In connection with this, Greene et al. (1978) have found that where 2'-O-methylation is concerned, interferon induction correlates with the degree of "clustering" of ribose residues. Thus, in a sample with a high percentage of 2'-OMe substitution, if there is a non-random distribution of these species and sequences of ribose residues of 6-12 base pairs exist with relatively high frequency, then this will be a better inducer than a sample with the same degree of substitution but a random distribution of residues, since uninterrupted runs of unsubstituted ribose residues will occur with much less frequency.

In an interesting study in which the normal 3', 5' linkage of the ribopolymer was interspersed randomly with 2', 5' linkages, Pitha

and Pitha (1971) found complete abolition of interferon induction, although it was admitted that the preparations were of relatively low molecular weight.

Collectively, these results appeared to show that, while the 2'-OH was important in some way, inducing ability was not necessarily affected by a high degree of substitution. Thus, while there might be a specific receptor determinant for the 2'-OH (Colby, 1971), it seemed more likely that destruction of the interferon inducing capability might be due to either (a) an alteration in the ribose-phosphate backbone conformation due to isomerisation of the sugar, or (b) alteration in the physical characteristics at the 2'-position, e.g. size, charge, or a combination of these. This view has been strengthened considerably with the recent finding that a double stranded $I_n.C_n$ complex, in which the 2'-OH of the inosinic acid strand has been entirely substituted by the azido group, is a potent interferon inducer (De Clercq et al., 1978b). (The efficacy of the 2'-chloro substituent in the same I_n strand as an interferon inducer was also reported briefly (Ikehara et al., 1977; De Clercq, 1977)). On the other hand, the complex of this 2'-azido inosinic acid with $(hr^5C)_n$ was not an inducer in the fibroblast cultures used, a rather surprising result in view of the high activity of $I_n.(hr^5C)_n$ as an inducer (Colby and Chamberlin, 1969) and all the more so since the $(z^{2'}I)_n.(br^5C)_n$ reacted well with antibodies directed against double-stranded RNA (see below).

As more detailed studies on structure-function relationships in synthetic polynucleotide interferon inducers have been performed, it has become apparent that the four criteria above probably only represent isolated aspects of a much more critical structural determinant, namely the overall conformation of the double stranded inducer.

Many polynucleotide complexes have been synthesised in which structural modifications of the heterocyclic base have led to varying degrees of activity as inducers (Pitha and Hutchinson, 1977; Torrence and De Clercq, 1977). In no case was there any obvious structural

change that could be said to result in a predictable shift in inducing ability. The position has become clearer, however, with the circular dichroism study of Bobst et al. (1976). The complexes $(c^7A)_n.U_n$ and $(c^7I)_n.C_n$, in which substitution of N-7 for carbon has been accomplished, display strikingly different activities, in that the former is a non-inducer whilst the latter is a highly active inducer. Inspection of the C.D. spectra and comparison with that for $A_n.U_n$ and $I_n.C_n$ shows that the two duplex analogues display altered spectra; in particular the major structural change was determined to be an increase in the positive base tilt for $(c^7A)_n$ and a small decrease of the same parameter in the case of $(c^7I)_n$. Halogenation of the 5'-position of the pyrimidine strand had no effect on the C.D. and this correlates well with the unaltered inducing capacity of all 5'-halogenated complexes studied (except for $(z^{2'}I)_n.(br^5C)_n$ above). The conformational effect of the change in the purine ring is, however, of critical importance, enhancing the concept that it is the overall spatial and steric organisation of a double stranded RNA that is the determinant of interferon induction. Thus, in cases where base substitution or alteration leads to inactive interferon inducers, it is most likely that the cause is an alteration of the duplex conformation sufficient to account for non-recognition by the putative ds RNA receptor.

Another relevant observation was made by Carter et al. (1972) who studied a series of duplexes in which a degree of base mis-matching had been introduced. Polymers in which Urd residues had been introduced into the I_n strand, e.g. $(I_{21}U)_n.C_n$ and $(I_{39}.U)_n.C_n$ were totally inactive as inducers, whilst introduction of Urd residues into the C_n strand produced a progressive decline in interferon inducing activity as the percentage of Urd was raised. At low percentages of Urd, e.g. in $I_n\ (C_{22}.U)_n$ and $I_n\ (C_{13}.U)_n$, interferon induction was, however, almost as good as with $I_n.C_n$ itself, as was the activity of $I_n\ (C_{20}.G)_n$ (with a low percentage substitution of Guo

residues). These observations appear to be explained by the concept of looping-out of the mis-matched residues in the non-inducers. This extrahelical loop may interfere, through steric interaction, with the proper alignment or approach of the intact unaltered helical segment of the duplex with the receptor. This theory is supported by practical experience obtained with a variety of mis-matched bases (Lomant and Fresco, 1975). Pyrimidine-pyrimidine oppositions such as U.C have never been observed to base pair, whilst most purine-pyrimidine oppositions can take up a form of intrahelical base pairing. In particular, in the above case the I-U opposition represents one of the Crick "wobble" pairs (Crick, 1966) and are known to exist intrahelically (Wang and Kallenbach, 1971). The I-G opposition in the I_n $(C_{20}.G)_n$ duplex was thought to be a case of extrahelical looping-out and hence an exception in terms of its activity. It seems more than likely, however, that under the conditions of ionic strength used, the low percentage of Guo could be accommodated intrahelically without distortion of the helix conformation, and such a probability has been foreseen by Topal and Fresco (1976a, 1976b) who showed the possible existence of G.G, A.G and I.G base pairs. The conclusion from this study is that those duplex inducers which can accommodate odd residues within their defined helical structure are likely to remain effective, whilst those which are forced to take up "helices with loops" will no longer be active. This again points to a critical overall helical conformation as the prime determinant for interferon induction. It is of some interest to note that those mis-matched duplexes which are good interferon inducers are also much less resistant to nucleases than was previously thought to be necessary since mis-matched bases are likely to "breathe" with a much higher degree of frequency than normal Watson-Crick base pairs. This property has been noted with very interesting results in trials of these polymers in mice (Ts'o et al., 1976; Carter et al., 1976). Good titres of interferon were obtained, but the toxic side effects normally associated with induction by $I_n.C_n$ were greatly reduced, and

this was put down to their relatively greater rate of destruction by serum nucleases. Since it has been shown that only very short exposure times are necessary for induction of an anti-viral state by synthetic ds RNA (De Clercq et al., 1971; Pitha et al., 1972), the high nuclease resistance suggested as a necessary criterion for activity is seen not to be entirely tenable.

It was hypothesised at an early stage that the receptor for ds RNA interferon inducers was likely to be a protein (Colby and Chamberlin, 1969), since a nucleic acid receptor would not be compatible with the variety of base sequences that are effective inducers. Further reasoning along these lines led Stollar's group to investigate the antigenicity of a series of inducers and non-inducers of interferon, since the antibodies elicited would be protein receptors for the ds RNA's. The results have provided ample confirmatory evidence that interferon inducers display special spatial conformations, and that this is probably the basis for their activity (Johnston et al., 1975; Johnston and Stollar, 1978). Thus, antibodies elicited against active interferon inducers show only weak cross-reactivity with non-inducer ds RNA's. The antibody systems are like interferon induction in that recognition depends on secondary structure and the nature of the sugar-phosphate backbone, and not on a specific base content or sequence of the ds RNA. Both strands of the RNA are recognised since ss RNA did not cross-react and changes in either or both strands led to severe decreases in reactivity. It is apparent then, that these antibody systems provide some of the best evidence so far that specific receptors for the ds RNA inducers of interferon probably exist at the cellular level.

4.1.3 The interaction between inducer and the cell

Critical to the understanding of the induction of interferon by ds RNA is a knowledge of the mechanism of triggering of the response and its location, be it in the cytoplasm, nucleus or at the cellular membrane. One of the earliest attempts to gain basic information

on this interaction were the experiments of Bausek and Merigan (1969). In the period that has followed the publication of these results, very little real progress can be said to have been achieved.

Bausek and Merigan incubated human fibroblast cells at 4^{o} in the presence of $I_n.C_n$ as inducer. If the cells were washed thoroughly and treated with RNase A at concentrations sufficient to destroy the added $I_n.C_n$, then no interferon induction was observed when the cells were subsequently warmed to 37^{o} C. In the absence of RNase treatment, good induction was observed, indicating not all of the inducer could be displaced by washing of the cells at 4^{o}. If, however, the cells, after treatment with $I_n.C_n$ at 4^{o}, were allowed to warm up to 37^{o} and then treated with RNase at various times postwarming, it was found that the development of an anti-viral state occurred rapidly if the treatment with RNase was delayed by only a matter of minutes. Thus, there appeared to be evidence for a temperature-dependent step in the induction of interferon, whereby inducer was susceptible to the action of RNase at 4^{o} but not after very short incubation at 37^{o}. Such a result suggested that penetration of $I_n.C_n$ could occur at 37^{o} leading to the induction of interferon, but at 4^{o} penetration was impossible and all inducer could be destroyed. To verify if this was the case, Bausek and Merigan (1969) then looked at the association of radioactive $I_n.C_n$ with the fibroblasts after the various treatments. It was found that after extensive washing at 4^{o}, only 0.2% of the initial inducer was still cell-associated. RNase treatment reduced this by a further 40-fold, but there was no detectable difference in the levels of inducer in cells which were treated with RNase immediately subsequent to the 4^{o} incubation compared with cells which were warmed to 37^{o} and given sufficient time to induce interferon before treatment with the RNase. It was concluded that only a fraction of the bound polymer was actually bound to sites important for triggering the interferon response. The location of these sites was not resolved, although the temperature-dependence suggested that penetration of the inducer might be necessary.

Autoradiographic analysis of rabbit kidney cells treated with ds RNA preparations showed that within 30 minutes at 37°, entry of the polymer could be detected, with high concentration in the region of the nucleolus developing (Prose et al., 1970). The polymer was also seen in the vicinity of projecting cell processes and in cytoplasmic vacuoles near the cell surface. Both results suggest uptake of the ds RNA by endocytotic mechanisms. The establishment that uptake of these polymers by cells was possible does not, however, answer the question as to whether uptake is necessary for interferon induction.

The non-specific nature of the association of the ds RNA inducer with the cell was further demonstrated in a series of experiments by De Clercq. No correlation was found between the amounts of ds RNA bound to human skin fibroblast cells (both inducers and non-inducers used) and the amount of interferon produced (De Clercq et al., 1972). Further, when a variety of cell lines (some inducible, others non-inducible) were tested with a fixed quantity of $I_n.C_n$, again no correlation between the amount bound and interferon produced was observed (De Clercq et al., 1972; De Clercq and De Somer, 1973). A similar result was observed for non-inducer DNA/RNA hybrids (Colby and Chamberlin, 1969).

When anti-bodies, raised against $I_n.C_n$, were added to cells which had been incubated with $I_n.C_n$ for extended periods at 4°, no anti-viral effect was seen when the cells were subsequently warmed to 37° (Vengris et al., 1975). Conversely, only very brief contact with the cells at 37° is required before addition of anti-serum for an anti-viral state to develop (as little as one minute). Longer periods of contact with the inducer are required (5-10 minutes) for assayable amounts of interferon to be produced. Again, there is indication of a temperature-dependent step.

Investigation of the binding of polynucleotides to human fibroblasts by Johnston et al. (1976) yielded results complementary to those of

Bausek and Merigan (1969). Non-specific binding of $I_n.C_n$ to these cells at 4^o could be decreased by up to 60% by washing the cells with 1 M sodium chloride at room temperature, or by incubation with a single-stranded polynucleotide such as C_n at 37^o. Interferon induction was also abolished by both these treatments and it was concluded that after 4^o incubation, the inducer duplex remains accessible, and is physically displaced from the membrane by the subsequent treatments. The inhibition of the interferon response by polynucleotide washing at 37^o C appeared to have a different basis to that observed by De Clercq et al. (1974a), who proposed in their experiments that an inactive triple stranded complex was formed. In the studies of Johnston et al. (1976), no triplex formation was possible since the three strands involved were not complementary. In cases where triple strand formation was not possible, De Clercq et al. (1974b) showed a normal interferon response ensued; the major difference in the studies was, however, the fact that De Clercq et al. (1974b) used 37^o incubation throughout, whilst Johnston et al. (1976) used a 4^o incubation. This supports the physical displacement hypothesis in view of the known accessibility of inducer after 4^o incubation (Bausek and Merigan, 1969).

Sequential administration of single-stranded polynucleotides results in as great or greater interferon inducing capacity, and it is believed that the single strands re-unite at the cell surface to form active double stranded inducer (De Clercq and De Somer, 1971; De Clercq and De Somer, 1972). This duplex appears to be more firmly bound than usual since insensitivity to ribonuclease (De Clercq et al., 1973) and salt washing and/or single stranded polynucleotide washing (Johnston et al., 1976) was observed. Whether the observation means that some $I_n.C_n$ has become internalised and hence able to induce interferon is not an interpretation made possible from these results, however.

As noted above, triplex formation at the cellular level inhibits the induction of interferon (De Clercq et al., 1974a). This inhibition occurs not only when a homopolymer is added to the cells in a mixture with a duplex inducer, but also when cells are incubated with the single stranded homopolymer for 1 hour before addition of the active duplex. This latter result is surprising in view of the fact that all non-specifically bound single strand is washed out before addition of the active duplex in very large excess. The implication is that single strands can bind to the double strand receptor, and are not displaced but form triplexes at the receptor site on addition of the double strand. On the other hand, the single strand is not inhibitory per se since good induction occurs when an active duplex is added to a system pre-incubated with a single stranded polymer that cannot form the complementary triplex (De Clercq et al., 1974b). Thus it seems that all classes of polynucleotides, single strand, duplex and triplex can bind to the receptor site. Single strands must be fairly loosely and reversibly bound whereas triplexes are inhibitory to subsequent treatment with active duplexes and must therefore bind tightly. Active ds RNA probably effects interferon induction by triggering a response that is exquisitely sensitive to the overall conformation of the helix.

4.1.4 Location of the double stranded RNA receptor site

One may postulate two locations for the ds RNA receptor in analogy with the known disposition of such sites for various hormones. Either the inducer can bind to a selective site on the cell surface and trigger the interferon response from there, e.g. via a "second messenger" mechanism, or it may be necessary for penetration to occur leading to productive binding at an intracellular receptor. It is of some interest to examine the evidence for either mechanism.

Pitha's research group has performed a number of investigations that purport to show that surface features are important in the

induction process. In the presence of moderate concentrations of the plant lectin, concanavalin A, the interferon response of human fibroblast and mouse 3T3 cells in response to $I_n.C_n$ was lowered 3 to 4-fold (Harper and Pitha, 1973). Such inhibition was, however, found to be independent of the extent of polynucleotide binding, although, at very high concentrations of the lectin, some lowering of the binding was noted. It should be pointed out, however, that the extent of the inhibition was not so marked, and certainly the interpretation that the Con A bound to a receptor vital for interaction with a ds RNA should be treated with caution. Besides its binding to glycosylated surface residues, Con A has also been shown to inhibit phagocytosis (Berlin, 1972) and virus penetration by non-specific uptake (Lonberg-Holm, 1975).

Studies of a similar nature employing enzymatic treatment of human fibroblast and mouse 3T3 cells prior to induction with $I_n.C_n$ gave qualitatively similar results (Pitha et al., 1974). Thus, interferon titres were reduced, this time by up to ten-fold, but certainly not abolished after pre-treatment with either neuraminidase or phospholipase C. Treatment with trypsin gave rather variable effects, but in general no significant reduction in interferon induction was observed. Again, conditions in which interferon induction was decreased did not affect the extent of binding by $I_n.C_n$ in analogy with the experiments with Con A (Harper and Pitha, 1974). It can be concluded that surface components that may be important either for binding or catalytic functions in interferon induction are modified by these treatments. On the other hand, such surface components may only be involved in non-specific transmission of ds RNA's into the cell. It might be noted that with only slightly higher concentrations of neuraminidase the cells lysed, so that drastic events were probably occurring. Similar studies performed in human fibroblast and mouse L cells have failed to disclose any inhibitory effect (T.K. Bradshaw, personal communication).

A direct way of testing whether induction could take place solely from the surface of the cell was to insolubilise the inducer by attachment to a solid support, and then look for interferon production after brief contact with the cells. This has been approached in a number of ways, amongst which may be mentioned attachment of $I_n.C_n$ to activated Sepharose (Taylor-Papadimitriou and Kallos, 1973; Bachner et al., 1975), Sephadex and cellulose (Pitha and Pitha, 1973), UV-induced attachment of $I_n.C_n$ to cellophane (Hutchinson and Merigan, 1975) and insolubilisation of $I_n.C_n$ by attachment to red blood cells (De Clercq and De Somer, 1974). In all of these reports (except one), a small but finite amount of leakage of $I_n.C_n$ from the solid support may well have accounted for the interferon induction seen in each case. Taylor-Papadimitriou and Kallos (1973) reported good interferon induction with their Sepharose-bound $I_n.C_n$, coupled with extreme stability of the preparation to alkali and nucleases. In view of the uniformly negative nature of the other results in respect of stability of $I_n.C_n$ under physiological conditions, this report must be treated with some reserve. To explain the stability of the preparation to alkali, it would be necessary to postulate that every base had become attached to the insoluble support.

A rather large number of studies has produced a good deal of circumstantial evidence that interferon induction is enhanced greatly by transport of the ds RNA inducer into the cell and hence, by implication, that penetration by the polymer is necessary for activity.
(a) In an extensive series of studies, Schell (1971) showed that Ehrlich ascites tumour cells took up significant amounts of ds RNA after incubation either at 0° or 30°. The cells were treated exhaustively with nucleases, sufficient to degrade the nucleic acids many times over, but a proportion of acid-precipitable counts remained cell-associated. It was also shown (by double-labelling) that $A_n.U_n$ was transported intact into the cell, but in the case of

$I_n.C_n$, strand separation occurred. This latter result has been disputed by Pitha et al. (1974) using double-labelling in human fibroblasts. In some cells which are very sensitive to the effect of ds RNA, intact $I_n.C_n$ may be recovered in homogenates after exposure to the inducer (De Clercq and Stewart, 1974). These results certainly show that cells are able to absorb significant quantitites of $I_n.C_n$ into the cytoplasm.

(b) Complexation of ds RNA with basic substances such as DEAE-Dextran has been shown to potentiate interferon production in many cases in vivo or in vitro (see Torrence and De Clercq, 1977). In most cases cell association of the inducer is much higher as a result of complexation, and this appears to be the basis for the effect (Pitha and Carter, 1971; Bausek and Merigan, 1969). However, it is not clear whether this cell association represents increased penetration of the inducer or just increased cell surface density. The fact that electroneutral complexes of DEAE-Dextran and $I_n.C_n$ are the most potent inducers (Pitha and Carter, 1971) suggests that these might be better able to penetrate the hydrophobic membrane.

(c) Meager et al. (1978) showed a similar potentiation of interferon production when high concentrations of calcium ion were introduced into an induction of various human cell lines by $I_n.C_n$. The same result was later observed in L cells (Booth and Borden, 1978). It has been noted previously that Ca^{2+} caused aggregation of adenovirus 5 DNA (Graham and Van der Eb, 1973) and increased uptake of the DNA into the cells, as shown by the 100-fold increase in plaque incidence. The interferon potentiation might be due to aggregation of ds RNA on the cell surface, but it is also likely that highly increased uptake is occurring.

(d) The polyene macrolide antibiotic, amphotericin B, increases interferon production in L cells induced with $I_n.C_n$ by 10 to 100 fold (Borden et al., 1978). The treatment was effective either before, during or several hours after the incubation with the ds RNA. It was

notable that the polyene did not appear to bind to the inducer, nor was there any increase in the cell-association of inducer after treatment. In view of the well-documented ability of polyene macrolides to bind to sterols in the cell membrane and increase permeability of such membranes, it seems likely that amphotericin B stimulated interferon production by increasing the cell penetration of the $I_n.C_n$ inducer.

(e) When $I_n.C_n$ was entrapped in large unilamellar lipid vesicles, both the uptake of labelled $I_n.C_n$ and the titre of interferon produced in mouse L cells and human fibroblast cells was increased (Mayhew et al., 1977). Such lipid vesicles are incorporated into the cell by either fusion or endocytotic mechanisms (Poste and Papahadjopoulos, 1976) and therefore the interferon result seems positively correlated with an increased intracellular concentration of inducer.

These studies have not yet been able to answer definitely the question of the location of the ds RNA receptor, but in general the evidence favours the concept of penetration into the cell by isolated ds RNA as a pre-requisite for interferon induction. It should be noted this conclusion says nothing about viral induction (but see discussion).

A new approach to the detection of possible membrane receptors for ds RNA was investigated in the present research. The undoubted usefulness of the photoaffinity labelling method in identifying important macromolecule-receptor associations (see Chapters One and Three) was considered to be an interesting and potentially important way of approaching the problem described. Since it had been possible to prepare synthetic double stranded $I_n.C_n$ complexes containing a small percentage of photolabile ^{8}azino residues (Chapter Two), their potential as inducers and as photoaffinity labels in the interferon system was investigated. The results of this work together with important new observations on the nature of the interaction of highly responsive cells with interferon inducers are reported below.

4.2 Materials and Methods

4.2.1 Materials

(a) Human cell lines used were:

(i) Human foreskin fibroblasts (HFF)

(ii) An osteosarcoma cell line, MG63, obtained from Dr. A. Billiau, Rega Institute, Leuven, Belgium (Billiau et al., 1977).

(b) Foetal calf serum was from Sera-Labs., Crawley Down, Sussex, U.K.

(c) Polynucleotides were a product of PL Biochemicals, Milwaukee, Wisconsin, U.S.A.

(d) Benzylpenicillin and streptomycin were obtained as an admixture, Crystamycin, from Glaxo Laboratories, Greenford, U.K.

(e) ^{198}Au (colloidal suspension, 10 mCi/mg Au) and [^{3}H]-uridine (26.4Ci/mmol) were from the Radiochemical Centre, Amersham.

(f) [^{125}I]-C_n was kindly prepared by Mrs. L. Harper, using the method of Commerford (1971).

(g) (z^{8}I, [^{3}H]-I)$_n$.C_n was prepared from z^{8}IDP and [^{3}H]-IDP as described in Chapter Two for the unlabelled material.

4.2.2 Cell culture

Cells were cultured in the Glasgow modification of minimal essential medium supplemented with non-essential amino acids, containing foetal calf serum (10% v/v), benzylpenicillin (100 units/ml) and streptomycin (100 µg/ml). Incubation was at 37° in a 5% CO_2/air atmosphere.

4.2.3 Interferon assays

These were performed in HFF cells and were based on the method of inhibition of nucleic acid synthesis as described earlier (Atkins et al., 1971). The titres are given in research reference units.

4.2.4 Photolysis experiments

The light source used was the Hanovia 100 W compact arc lamp, as described in Section 2.2.2 but without the quartz lens. For photolysis experiments on cells in the presence of polynucleotide inducers,

the apparatus was as shown diagrammatically in Figure 4.2. The deflecting mirror was silvered quartz and was necessary since the lamp had to be operated in the horizontal position. The energy falling on the cell sheet was reduced by the presence of the lid of the plastic petri dish, and by the insertion of a variety of glass filters in the light beam, and its intensity was measured as described in Chapter Two. The temperature was maintained as desired by placing the petri dish on the cooling block of an LKB Multiphor (LKB Instruments, Croydon, U.K.) and pumping through water of the required temperature. Good thermal contact was achieved by ensuring that the underside of the petri dish rested on a thin film of water. Under these conditions, the temperature of the culture fluid in the dish never rose above 4^{o} when ice-water was circulated through the cooling block.

4.2.5 Interferon inductions

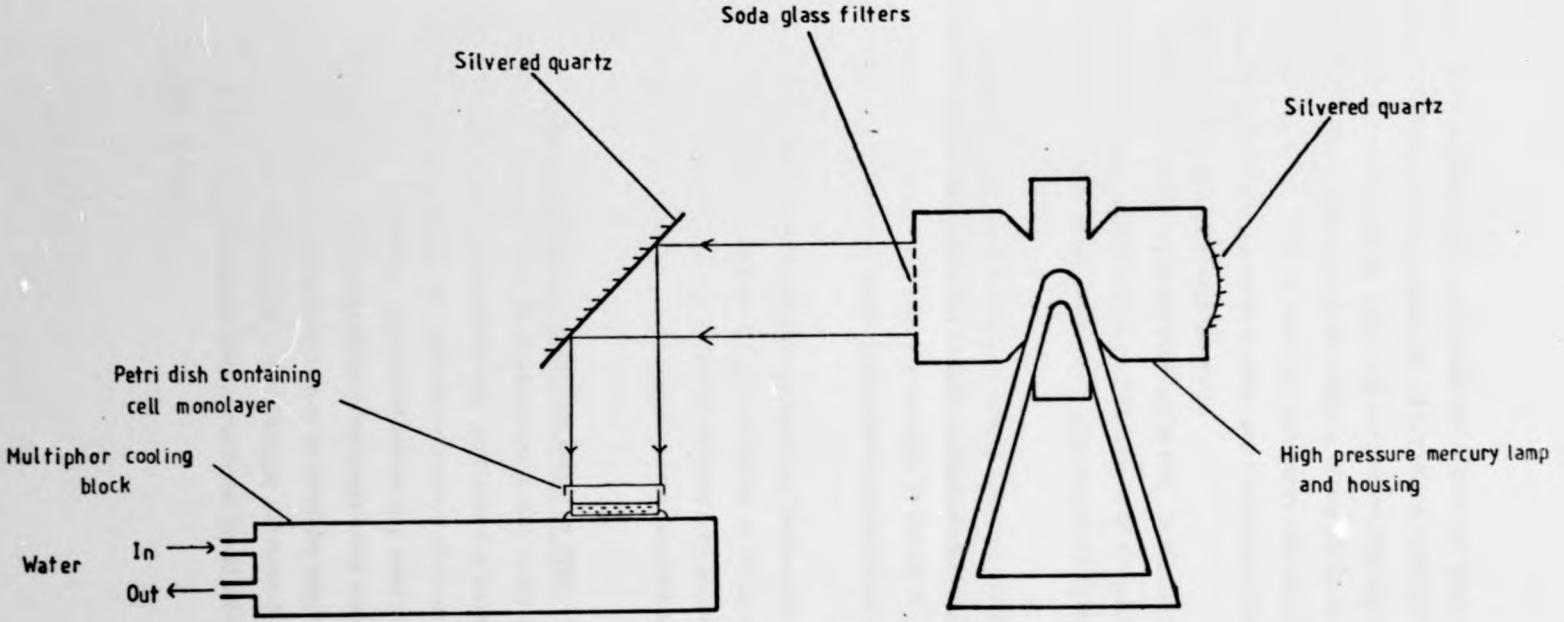
The basic procedure is outlined below. Where variations were used, these are noted in the relevant parts of the text or in the legends to Tables and Figures.

For a simple induction at 37^{o}, confluent monolayers of MG63 cells on 50 mm dishes were washed four times with serum-free medium (2 ml) and then incubated with $I_n.C_n$ solution (50 µg/ml in serum-free medium) for 1 hour at 37^{o}. The supernatant was drawn off, the cell sheets washed several times more with serum-free medium (2 ml) and finally incubated overnight in maintenance medium (i.e. containing 2% foetal calf serum, 2 ml). Fluids were collected and stored at -20^{o} until required for assay.

For inductions involving a 4^{o} incubation, the following general protocol was followed. Confluent monolayers of MG63 cells in 30 mm dishes were washed with serum-free medium (4 x 1 ml). A further portion of serum-free medium was added (1 ml) and the cells allowed to cool at 4^{o} for 15 minutes. The medium was replaced by cold (4^{o}) $I_n.C_n$ solution (0.72 ml, 50 µg/ml in serum-free medium) and incubated at 4^{o} for 1 hour. The cells were washed with

Figure 4.2

Diagrammatic representation of apparatus used in MG63 cell photolysis experiments


Soda glass filters
Silvered quartz
Silvered quartz
Petri dish containing
cell monolayer
Multiphor cooling
block
High pressure mercury lamp
and housing
Water
In
Out

serum-free medium (4 x 1 ml) and then incubated with a further portion of serum-free medium (0.72 ml) for 1 hour at 37°. Subsequent washing of the cells and incubation overnight was as described above, except that the volume of maintenance medium used was 0.72 ml. The smaller 30 mm petri dishes were necessary since they could be evenly and completely illuminated when used in photolysis experiments.

When inductions were carried out in PBS, cell washings and incubations were performed with PBS instead of serum-free medium. Overnight incubation was, however, still necessarily done in maintenance medium.

In experiments where a salt-washing step was performed, cells were incubated (after removal of the polynucleotide inducer) for 5 minutes with 1 M sodium chloride solution (1 ml) at 4°, and then washing and subsequent operations were performed as described above.

Before assay, the interferon-containing fluids were incubated for 1 hour at 37° with a mixture (20 µl) of RNase A (50 µg/ml) and RNase T_1 (300 units/ml) in order to destroy any polynucleotide remaining in the solution which might induce interferon in the assay cells.

4.2.6 Quantitation of polynucleotides bound to cells

The experiments (details of which are found in the appropriate legends to Figures and Tables) were performed in exactly the same manner as described for the interferon assays above at the appropriate temperatures. However, instead of incubating overnight for collection of the interferon-containing fluids, the cells were washed twice in cold 5% trichloroacetatic acid and once in absolute ethanol. Cell sheets were dissolved in sodium hydroxide solution (0.5 M, 1 ml) and samples were counted in the appropriate liquid scintillation counter or gamma counter.

4.2.7 Uptake of ^{198}Au by MG63 cells

The ^{198}Au suspension was diluted into the relevant solution (PBS or serum-free medium) to a final specific activity of 10 μCi/ml. Dishes of MG63 (50 mm) were washed as described above for inductions, and ^{198}Au solution was added (0.5 ml per dish, 5 μCi) for varying periods of time with incubation at 4°. Cell sheets were washed copiously with the relevant medium to ensure complete removal of non-specifically bound gold, and then the cell sheets were dried in a warm room. After dissolution of the cell sheets in sodium hydroxide solution (0.5 M, 2 ml), the radioactivity in the samples was measured.

4.3 Results and Discussion

4.3.1 Photolysis studies

As described in Chapter Two, the synthetic ribopolymer $(z^8I, I)_n$ in which z^8Ino residues comprise only 2.5% of the nucleotidic content, readily formed a 1:1 hybrid with C_n. Figure 4.3 shows the UV spectrum of this complex in 0.16 M sodium phosphate solution, pH 7.0, compared to a sample of $I_n.C_n$ annealed under identical conditions. The two spectra are virtually identical except for the slightly extended long wavelength absorption displayed by the azido complex, due to the z^8Ino residue. The hypochromicities at 245 mm were 31.2% and 33.9% for $I_n.C_n$ and $(z^8I, I)_n.Cn$ respectively. It was felt therefore that the azido complex would prove to be active as an interferon inducer. The results in Table 4.1 show the interferon yield after a simple induction at 37°. The azido complex is a fairly good inducer, but it is clear that its efficiency is much lower than that of $I_n.C_n$ since its dose-response has not reached a maximum even at 50 μg/ml, conditions that have saturated the $I_n.C_n$ induced interferon response (see below).

As a preliminary to experiments in which the properties of interferon induction as a function of photolysis of the azido complex were studied, the response of the cells to inducers applied at 4° was observed. Under these conditions it is assumed that the polynucleotides

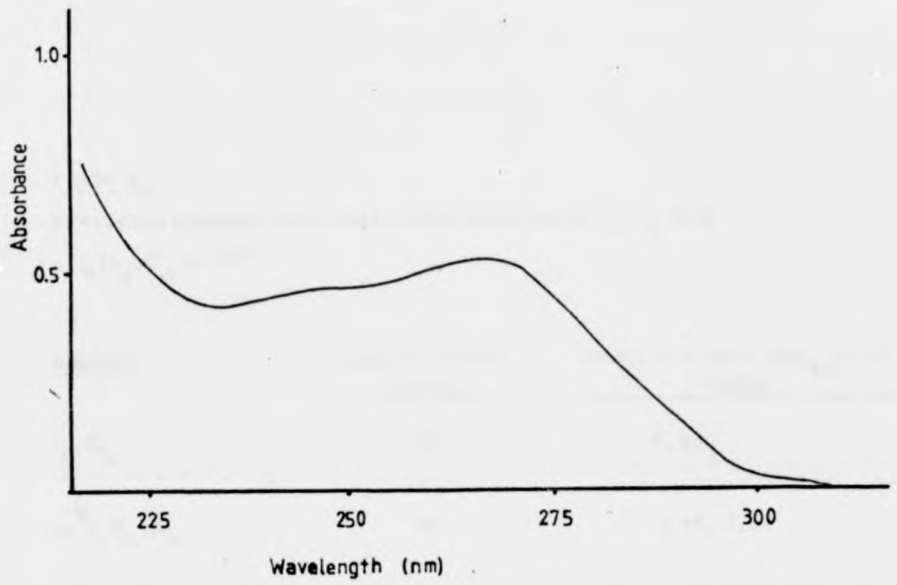
Figure 4.3

Ultra-violet spectra of 1:1 hybrid polynucleotides

(a) $I_n.C_n$

(b) $(z^8I, I)_n.C_n$ (containing 2.5% z^8Ino) recorded in 0.16 M sodium phosphate, pH 7.0

a)


1.0
0.5
Absorbance
225
250
275
300
Wavelength (nm)

b)

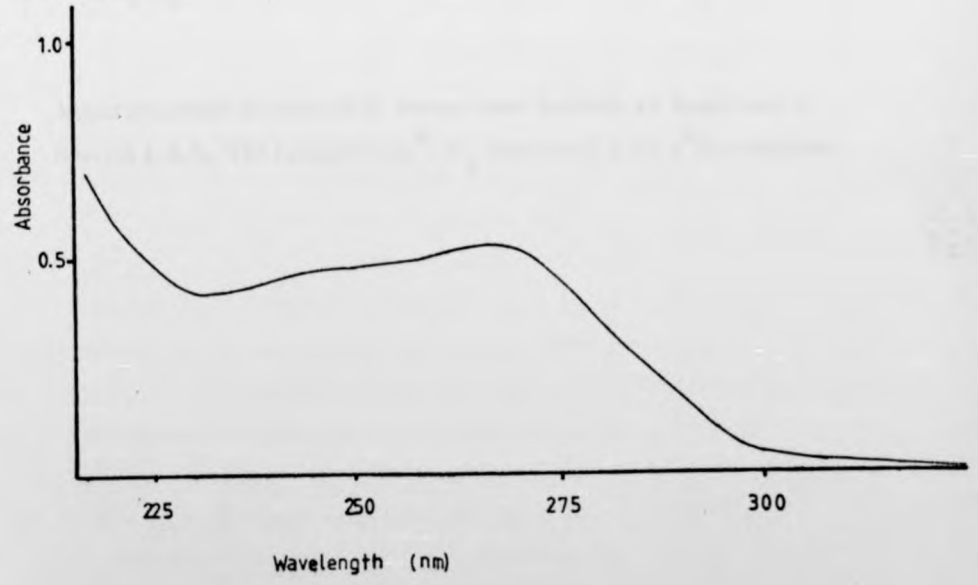

1.0
0.5
Absorbance
225
250
275
300
Wavelength (nm)

Table 4.1

Interferon titresof MG63 cells after induction by $I_n.C_n$ and $(z^8I, I)_n.C_n$ at 37°

Inducer	Concentration µg/ml	Interferon titre ($\log_{10}$U/10^6 cells)
$I_n.C_n$	50	3.45
$(z^8I, I)_n.C_n$	25	2.15, 2.39
$(z^8I, I)_n.C_n$	50	2.60, 2.68
$(z^8I, I)_n.C_n$	100	2.81

Inductions were performed in serum-free medium as described in Section 4.2.5. The sample of $(z^8I, \cdot$ contained 2.5% z^8Ino residues.

are only superficially bound (Bausek and Merigan , 1969; Vengris et al., 1975). The protocol used here involved incubation at 4^{o} for 1 hour, then thorough washing of the cells before incubation at 37^{o} for a further period. The results for $I_n.C_n$ are fully in accord with those of Bausek and Merigan (1969) in that sufficient double strand remains bound at 4^{o} for the temperature-dependent triggering of interferon induction at 37^{o} to be observed (see Table 4.2). Unexpectedly, however, the azido complex displayed very low activity under these conditions, whereas the titre of the $I_n.C_n$ complex appeared undiminished compared to a normal 37^{o} induction (Table 4.1). This was a novel observation and is discussed further below. What this result does prove, however, is that the induction of interferon by the azido complex at 37^{o} is genuine, and not due to a small amount of $I_n.C_n$ contaminant, since if this latter had been the case, induction after the $4^{o}/37^{o}$ treatment would also have been seen.

When the cells were photolysed for different periods in the presence of the ds RNA at 4^{o}, an increase in IF titre was observed in those cells treated with the azido complex, but not those treated with $I_n.C_n$ (Table 4.2). The obvious interpretation of this result, that the increase in IF titre is due to increased binding of the azido complex to the cell membrane by photolytically induced covalent links, was tested by performing further experiments, involving washing with 1 M NaCl (Johnston et al., 1976).

Figure 4.4 shows the change in UV spectrum of the hybrid $(z^{8}I, I)_n.C_n$ under the conditions of photolysis used, together with the wavelength cut-off characteristics of the filters. Decomposition of the azido groups appears to occur readily, causing a small but detectable change in the spectrum. It seems highly likely, therefore that nitrenes are being generated within complexes in contact with the cell surface.

As described in the introduction, Johnston et al. (1976) found that treatment of human fibroblast cell cultures, maintained at 4^{o} in the presence of $I_n.C_n$ with 1 M NaCl, abolished the interferon response of these cells when warmed to 37^{o} and also dislodged some 60% of the bound polynucleotide. Presumably, amongst this 60%, had been some

Table 4.2

Effect of photolysis on interferon titres in MG63 cells during induction by $I_n.C_n$ and $(z^8I, I)_n.C_n$ at 4^o

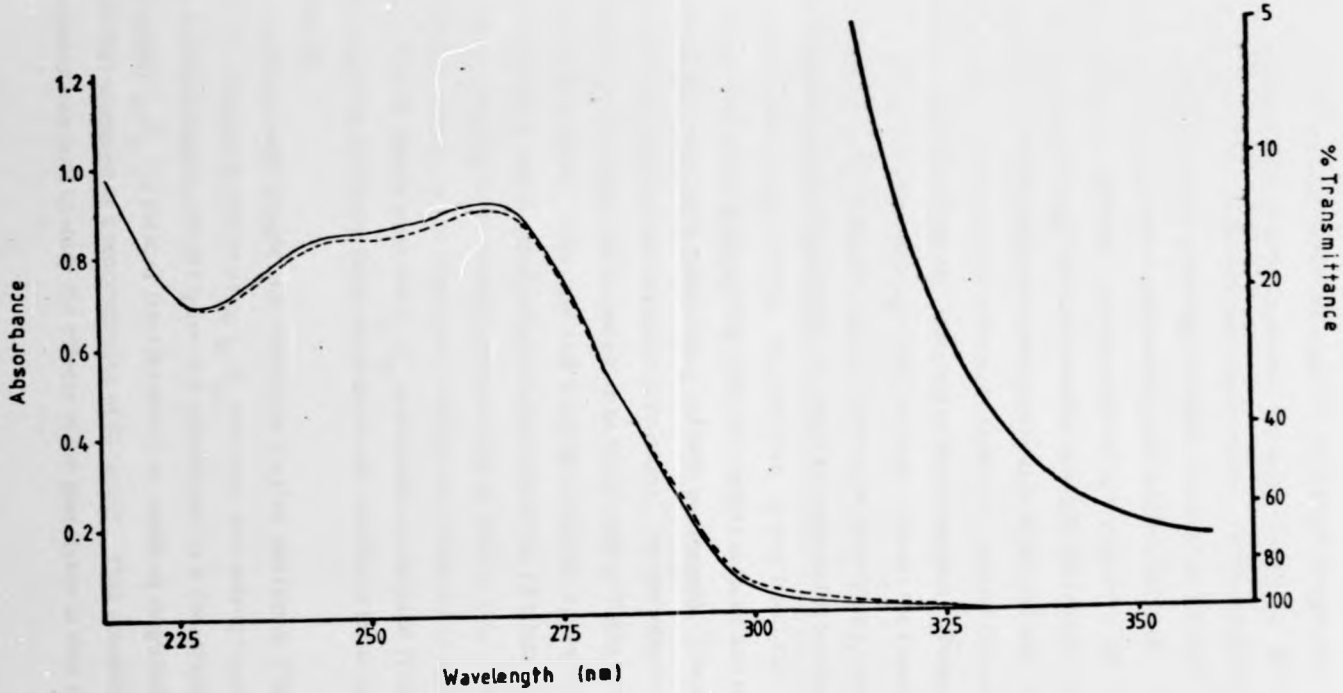
Inducer	Period of photolysis (min)	Interferon titre ($log_{10}U/10^6$ cells)
$I_n.C_n$	0	3.54, 3.57
$I_n.C_n$	5	3.62
$I_n.C_n$	15	3.57, 3.62
$(z^8I, I)_n.C_n$	0	1.02, 1.03
$(z^8I, I)_n.C_n$	5	1.16, 1.31, 1.37
$(z^8I, I)_n.C_n$	15	1.70, 1.94

Inductions and photolyses were performed in 30 mm dishes with polynucleotide (50 μg/ml) in serum-free medium at 4^o, and subsequent incubation at 37^o as described in Section 4.2.5. Light intensity incident at the cell monolayer was measured at 1660 erg/sec/mm^2. The sample of $(z^8I, I)_n$ contained 2.5 % z^8ino residues.

Figure 4.4

Ultra-violet spectra of 1:1 hybrid of $(z^{8}I, I)_{n}.C_{n}$ (as in Figure 4.3b) before (-----) and after (———) photolysis for 15 minutes at 4^{o}

The sample was contained in a 30 mm petri dish at 50 μg/ml in PBS and irradiated in the apparatus depicted in Figure 4.2. The wavelength characteristics of the filter combination (petri dish lid and soda glass) are also shown. Light energy measured at the surface of the PBS was 1660 erg/sec/mm^{2}


1.2
1.0
0.8
0.6
0.4
0.2
Absorbance
225
250
275
300
325
350
Wavelength (nm)
5
10
20
40
60
80
100
% Transmittance

polynucleotide bound at important sites for interferon triggering, be they receptor sites or sites important for internalisation. It was reasoned therefore, in the present experiments, that if photolysis of the azido complex was resulting in stable association between receptor and inducer, then a salt washing procedure would not dislodge this polynucleotide, and thus instead of a reduced yield of interferon the titre would be maintained or possibly increased. Such an argument would be untenable if internalisation of the inducer into the cytoplasm is important for interferon induction, since obviously the inducer would be covalently attached to the membrane. From Table 4.3 it is found that although salt washing reduces the response of the cells to $I_n.C_n$ induction (in the absence of photolysis), it does not completely abolish this activity as would be expected from the results of Johnston et al. (1976). In particular, it was found that after photolysis, the titres increased for both the normal inducer and the azido inducer, even after salt washing had been performed. These values at first sight appeared rather paradoxical, but the effects of the photolytic treatment are comparable to those seen in Table 4.2 for the azido inducer. Thus, the salt washing treatment appears to be having only a small effect which is counteracted by an effect of the photolysis, leading to an overall greater titre in both cases. It was of interest, therefore, to determine whether the observed lack of increase in IF levels when the $I_n.C_n$ system was photolysed (Table 4.2) was due to it already being saturated in its interferon inducing capability.

Conditions were sought under which the yield of interferon from a $4^o/37^o$ incubation scheme using $I_n.C_n$ induction was sub-optimal. The dose response curve of Figure 4.5 showed that at a concentration of 2 μg/ml $I_n.C_n$ the yield of interferon was an order of magnitude below that produced at a concentration of 50 μg/ml. This concentration of inducer was used to study the effects of the photolysis in this system.

Table 4.3

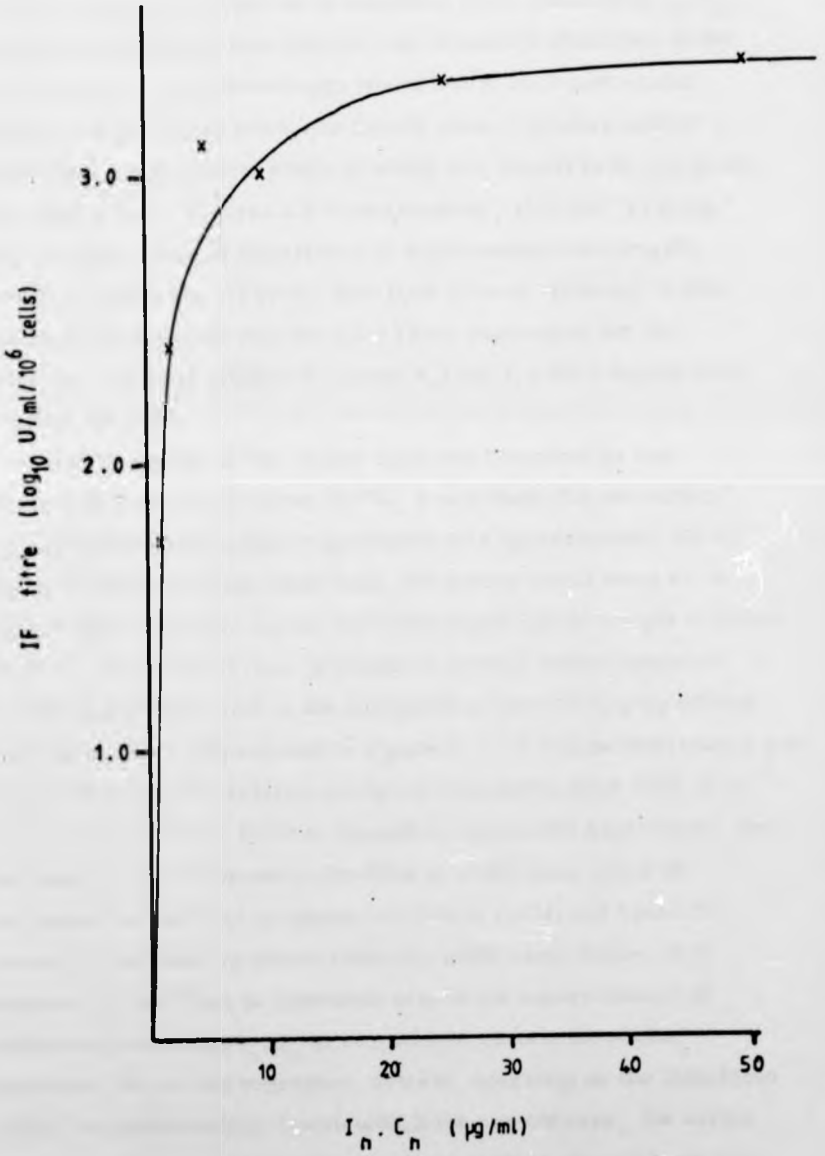
Effect of combined photolysis and sodium chloride washing on interferon titres in MG63 cells during induction by $I_n.C_n$ and $(z^8I, I)_n.C_n$ at 4°

Inducer	Period of photolysis (min)	Interferon titre (log_{10} U/10^6 cells)
$I_n.C_n$	0	2.80
$I_n.C_n$	5	3.05
$I_n.C_n$	15	3.60
$(z^8I, I)_n.C_n$	0	0.6
$(z^8I, I)_n.C_n$	5	1.25
$(z^8I, I)_n.C_n$	15	1.68

Conditions of induction and photolysis were as described in Table 4.2, except that incubation with sodium chloride (1 M, 1 ml) for 5 minutes was performed as described in Section 4.2.5.

Figure 4.5

Dose-response curve for interferon induction in MG63 cells by $I_n.C_n$. Induction was performed in serum-free medium at 4^o in 30 mm dishes as described in Section 4.2.5


3.0
2.0
1.0
IF titre ($\log_{10}$ U/ml/10^6 cells)
10
20
30
40
50
$I_n . C_n$ (µg/ml)

Mozes and Vilcek (1974) showed that pre-irradiation of cells for short periods with hard UV light (i.e. with energy peak at 254 nm) led to an increased production of interferon after induction by $I_n.C_n$. In the present study it was realised that attempted photolysis of the azide with light of this wavelength would lead to such undesirable effects, so that filters were specifically used to produce light of a wavelength, the biological effect of which was thought to be negligible (see Figure 4.4). Figures 4.6 shows, however, that this "priming" effect of light is also of importance in studies where wavelengths greater than 300 nm are used. Such light induced "priming" of the interferon response is thus the most likely explanation for the increases in titre presented in Tables 4.2 and 4.3 for induction with the azide polymer.

When the energy of the visible light was compared to that reported by Mozes and Vilcek (1974), it was found that the output/unit area used in the present experiments was approximately 500 to 1000-fold higher. On the other hand, the energy quanta were all in the 300 nm and greater region and hence might not be thought to affect the DNA. Setlow (1974) has produced an average action spectrum for the sensitivity of DNA to the mutagenic or other damaging effects of sunlight; this is reproduced in Figure 4.7. It will be seen that in the region of 300 nm, the relative biological sensitivity of the DNA is 10^{-2}-10^{-3} that at 260 nm. If this is the case in the present experiment, then the energy being absorbed by the DNA is of the same order of magnitude as that found by Mozes and Vilcek (1974) and hence the priming effect found is almost certainly of the same origin. It is assumed that the effect is somewhat akin to the superinduction of interferon produced by $I_n.C_n$ in response to certain metabolic inhibitors, in that the repressor system, operating on the interferon mRNA, is preferentially inactivated. In the present case, the active spectrum of the repressor gene may show greater sensitivity to long wavelength light compared to the interferon gene, by virtue of a different base content, or association with certain types of susceptible nuclear proteins.

Figure 4.6

The effect of light on the production of interferon in MG63 cells. Inductions were performed at 4^{o} with $I_n.C_n$ (2μg/ml) in serum-free medium as described (Section 4.2.5). Photolysis was performed in the presence of the inducing polynucleotide at 4^{o} C, using the filter combination of Figure 4.4. Light energy was 1660 erg/sec/mm^2

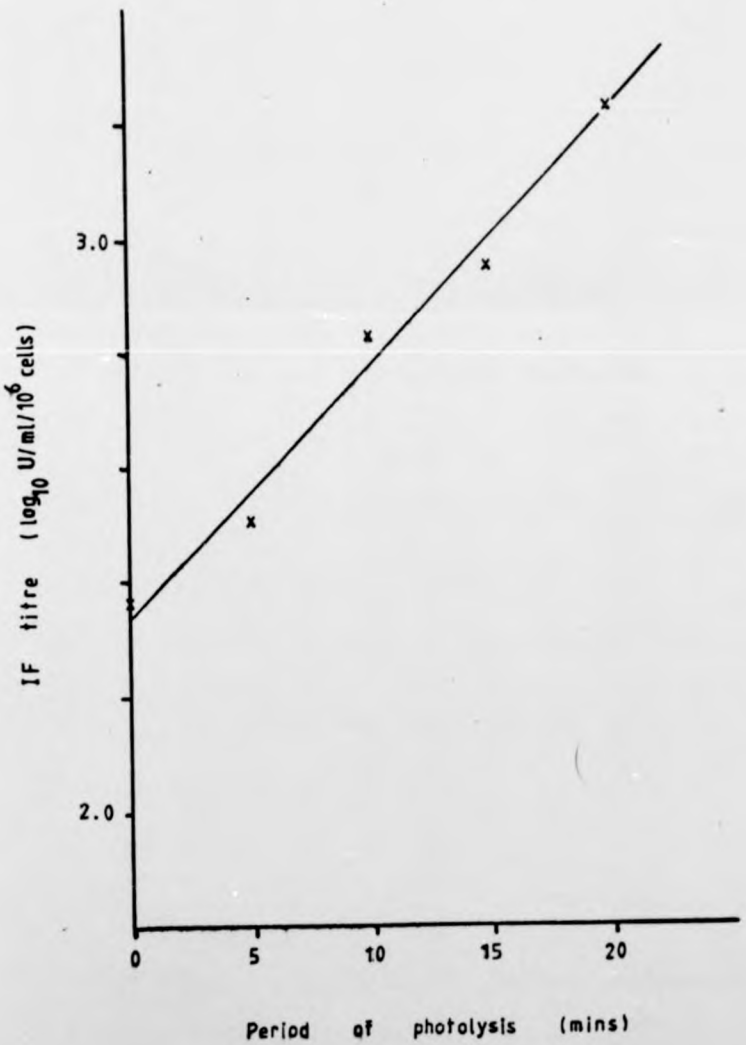
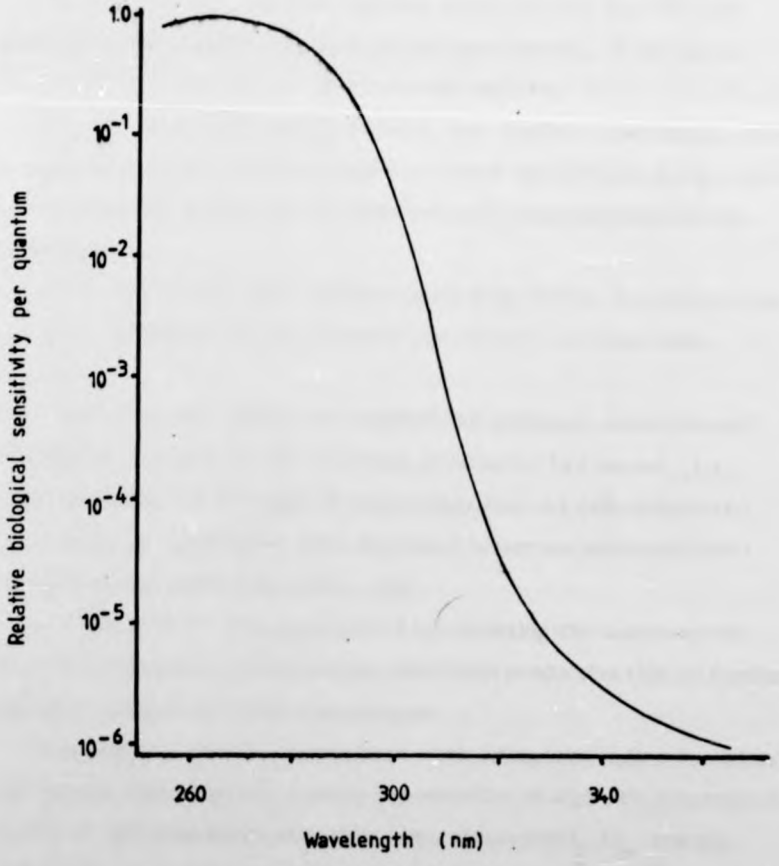

IF titre ($\log_{10}$ U/ml/10^6 cells)
3.0
2.0
0
5
10
15
20
Period of photolysis (mins)

Figure 4.7

Action spectrum of light-induced damage to DNA-containing systems (Setlow, 1974)


1.0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
Relative biological sensitivity per quantum
260
300
340
Wavelength (nm)

This is a most unfortunate result in two ways. Firstly, it might have been possible to abolish the "priming" effect by increasing the wavelength to which the cells were exposed. However, this would then be in regions where effective photolysis of the $(z^8I, I)_n.C_n$ inducer would not occur. Secondly, it means that the photoaffinity labelling approach on which this part of the investigation was based is not likely to be useful in its present form at least. To show that a photoaffinity label could be effective in helping delineate the questions of membrane receptors versus intracellular penetration of a ds RNA inducer, it is necessary to show that not only is the photolabile analogue an inducer, but that exterior manipulations (in this case photolysis) do not affect the system non-specifically. That this is not the case, however, is clear from the results.

Because of the non-specific effects, any further experiments aimed at detecting whether the supposed membrane receptors had been blocked by covalent attachment of the azido inducer would suffer from two problems:

(a) After the UV treatment, Mozes and Vilcek (1974) found that cells produced interferon for an extended period up to 28 hours after induction.

(b) Any attempt to determine whether the receptors were blocked would have to wait until all interferon production had ceased, i.e. after 28 hours, but it would be quite likely that the cell-associated azido polymer would have been degraded by serum and membrane-associated nucleases long before this.

It is because the only sure way of interpreting the usefulness of the technique is by way of assaying interferon production that no further approach along these lines was pursued.

However, the results of the above work brought to light some other unexpected observations, namely the inability of high salt concentrations to abolish the interferon response when induced by $I_n.C_n$, and the ambiguity observed in induction by $(z^8I, I)_n.C_n$ at 4^o or 37^o compared to $I_n.C_n$. These were investigated further.

4.3.2 The binding of $(z^8I, I)_n.C_n$ to MG63 cells

The interferon inducing ability of the $(z^8I, I)_n.C_n$ complex was investigated with a number of samples made at different periods during the research (z^8Ino content = 2.5%), but each time the same result was obtained, i.e. at 37° good activity was displayed at a concentration of 50 μg/ml, but after treatment at 4° followed by washing and subsequent 37° incubation, no yield of interferon was ever obtained. To determine whether this could be correlated with a lack of binding to the cell at 4°, tritiated $(z^8I, I)_n$ was synthesised from [^{3}H]IDP and z^8IDP, and the extent of binding of its hybrid with C_n to MG63 cells examined. This material was of a rather low specific activity (4.25×10^3 cpm/μg) and no significant counts were bound to cells after either 4° or 37° treatment. By contrast, however, a radioactive $I_n.[^{125}I]$-C_n preparation of the same specific activity as the azido complex gave significant results (Table 4.4), so that it was clear that a much reduced degree of cell binding was taking place with the azido complex.

To enable this low level of binding to be quantitated, a sample of the azido complex was prepared in which the C_n strand was iodinated, thus allowing a much greater initial specific activity to be achieved. The results of the binding of this sample are shown in Table 4.5, together with the respective interferon yields. The binding of the azido complex at 4° is an order of magnitude lower than that of $I_n.C_n$. At 37° the difference is less, but still 5-fold lower; on the other hand, a reasonable titre of interferon is produced. Interestingly, in the case of $I_n.C_n$, the extent of binding to the cells (0.1 - 0.2%) is very similar to that observed by Bausek and Merigan (1969) in human fibroblast cells, and by Johnston et al. (1976) in Flow 1000 fibroblasts.

Whilst it is clear that there is greatly reduced binding of the azido complex to these cells, it seems unlikely, when comparing the figures for 4° and 37° inductions that the small difference in amounts bound at these two temperatures (2.5-fold) is sufficient reason for, on the one hand, a reasonable interferon response, and on the other, a total absence of response.

Table 4.4

MG63 cell-associated radioactivity after treatment with low specific activity radioactive polynucleotides

Inducer	Incubation temperature (^{o}C)	Input counts bound (%)	Bound polynucleotide ($\mu g/10^6$ cells)
$(z^8I,[^3H]\text{-}I)_n.C_n$	4	0 †	0
$(z^8I,[^3H]\text{-}I)_n.C_n$	37	0 †	0
$I_n.[^{125}I]\text{-}C_n$	4	0.158	0.0568
$I_n.[^{125}I]\text{-}C_n$	37	0.218	0.0783

Experiments were performed with washed MG63 cells in 30 mm dishes at either 4^{o} or 37^{o}. Radioactive polynucleotide (50 μg/ml, specific activity 4.25×10^3 cpm/μg, 0.72 ml) in serum-free medium was incubated for 1 hour at the respective temperature, after which cell sheets were washed several times with proteins of serum-free medium (1 ml) until no further counts were released. Subsequent treatment of cell sheets was as described in Section 4.2.6. The sample of $(z^8I,[^3H]\text{-}I)_n$ contained 2.5% residues.

† counts not detectable above background

Table 4.5

MG63 cell associated radioactivity and interferon response after treatment with high specific activity radioactive polynucleotides

Inducer	Incubation temperature (°C)	Input counts bound %	Bound polynucleotide ($\mu g/10^6$ cells)	Interferon titre ($\log_{10} U/10^6$ cells)
$(z^8I, I)_n.[^{125}I]\text{-}C_n$	4	0.0166	0.006	< 0.5
$(z^8I, I)_n.[^{125}I]\text{-}C_n$	37	0.0418	0.015	2.6
$I_n.[^{125}I]\text{-}C_n$	4	0.173	0.0621	2.9
$I_n.[^{125}I]\text{-}C_n$	37	0.216	0.078	3.5

Inductions and incubations were performed in serum-free medium at a concentration of 50 μg/ml polynucleotide. Other conditions were identical to those of Tables 4.1 and 4.2 for induction, and to Table 4.4 for quantitation of radioactive polynucleotide bound. Specific activities of polynucleotides were: $(z^8I, I)_n.[^{125}I]\text{-}C_n$ (1.81×10^5 cpm/μg), $I_n.[^{125}I]\text{-}C_n$ (3.56×10^4 cpm/μg) and 0.72 ml was added to 30 mm dishes. The sample of $(z^8I, I)_n.[^{125}I]\text{-}C_n$ contained 2.5% z^8Ino residues.

In Chapter Two it was stated that the $(z^8I, I)_n.C_n$ complex probably existed at ambient temperature at least, as a 1:1 hybrid, with the z^8Ino residues constrained in an anti conformation by virtue of Watson-Crick base pairing. Thus, an overall double stranded helical geometry would be adopted with no looping out of z^8Ino residues due to base mis-matching. Carter et al. (1972) performed a study on $I_n.C_n$ complexes, in which a small percentage of U residues in the I_n strand had been substituted. It was found that $(I_{21}, U)_nC_n$ and $(I_{39}, U)_n.C_n$ were completely ineffectual as interferon inducers. Wang and Kallenbach (1971) had shown that pyrimidine-pyrimidine oppositions were incapable of base pairing, and hence the looping out of the U residues in these complexes was sufficient to cause a distortion of the helical geometry sufficient to be unrecognisable by the ds RNA receptor. It appeared that this receptor was particularly sensitive to such looping since in $(I_{39}, U)_n.C_n$ the percentage of base mis-match was only 2.5%. This figure is the same as the percentage substitution by z^8Ino in the I_n strand, and it was considered possible that a similar looping out structure was adopted leading to an absence of induction at 4^o.

Bobst et al. (1976) showed that C.D. spectra of ds RNA inducers and non-inducers were sufficiently different to account for a conformational basis of recognition as an inducer. Thus, the possibility of a helix structural change occurring between 4^o and ambient temperature was investigated for $(z^8I, I)_n.C_n$ by C.D. (Figure 4.8). Whilst the overall spectrum at the two temperatures is qualitatively similar (virtually identical for $I_n.C_n$), the magnitude of the Cotton effects was decidedly smaller at 4^o in the case of $(z^8I, I)_n.C_n$. This is an unusual observation since reduction in Cotton effect intensity is normally associated with decreased stacking activities caused by an increase in temperature (Tinoco and Cantor, 1970). However, the likely explanation is that the sample at 4^o had been annealed by cooling directly from 70^o and kept at 4^o continuously thereafter. Under these conditions, base mis-matching may well have occurred since no time for equilibration to give a fully base-paired structure was allowed.

Figure 4.8

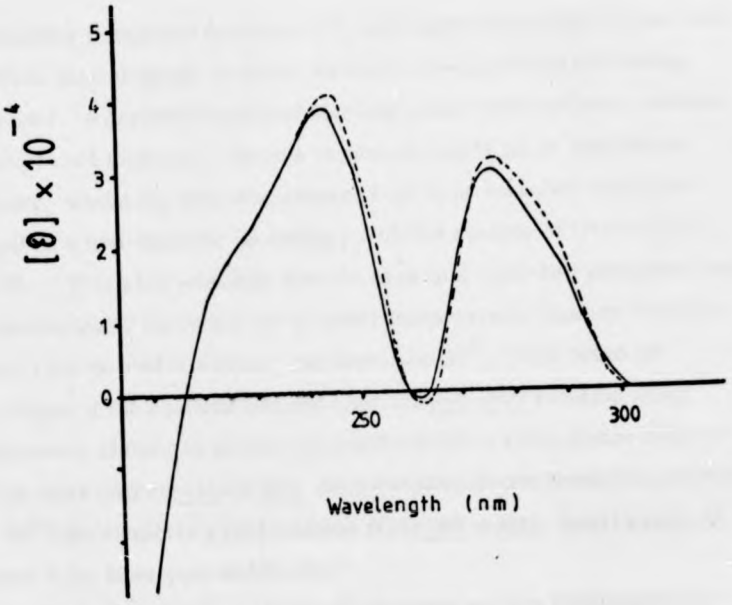
Circular dichroism spectra of 1:1 hybrid polynucleotides

(a) $I_n.C_n$ at 4^o (——) and at 25^o (------)

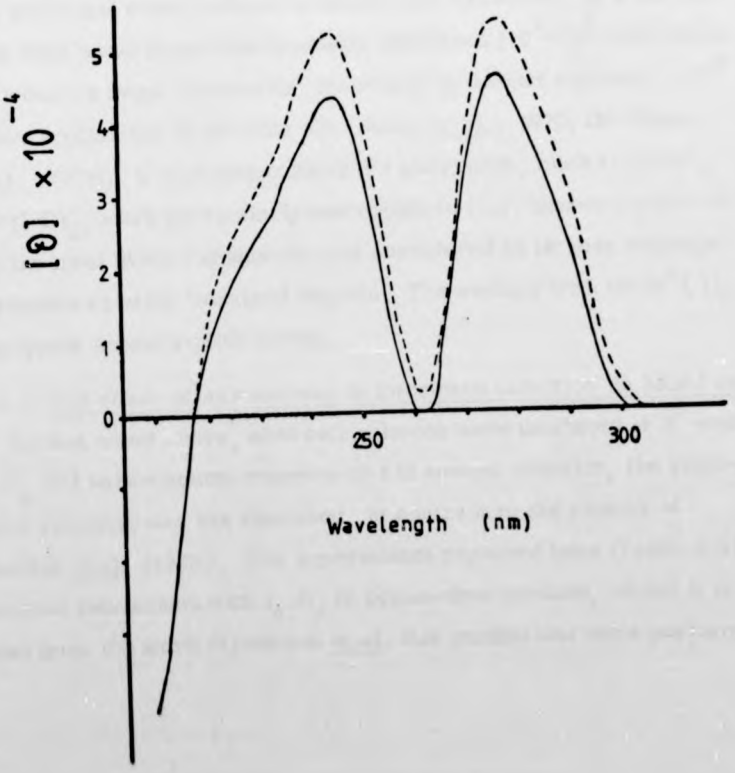
(b) $(z^8I, I)_n.C_n$ at 4^o (——) and at 25^o (-------)

Spectra were recorded in phosphate buffered saline

a)


[θ] x 10⁻⁴
5
4
3
2
1
0
250
300
Wavelength (nm)

b)


[θ] x 10⁻⁴
5
4
3
2
1
0
250
300
Wavelength (nm)

At ambient temperature and at 37°, sufficient flexibility is generated to allow base slippage to occur with all non-paired regions being removed. A greater degree of stacking would lead to Cotton effects of increased intensity. Such a structure would be an interferon inducer, whilst the low temperature base mis-matched structure would be a non-inducer by analogy with the results of Carter et al., (1972). It is also possible that the base mis-matched structure may be intrinsically more stable at lower temperature than the Watson-Crick base paired structure, but less so at 37°. This could be envisaged if the equilibrium constant for syn-anti rotation were sufficiently shifted to favour syn conformations over stable Watson-Crick base pairing via an anti conformation at the lower temperature. At 37° the relatively rapid rotation from syn → anti could result in stable fully base-paired helixes.

Finally, mention may be made of some results obtained by De Clercq that may have some bearing on the observation reported here. The anti-viral effect induced in human skin fibroblast, by a variety of ds RNA's was found to be markedly increased (10^2-10^5-fold) when the inducers were "thermally activated" by a short exposure to 37° before application to the cells (De Clercq et al., 1970; De Clercq et al., 1971). It was noticeable that copolymers, such as $(A\text{-}U)_n$ and $(I\text{-}C)_n$, were particularly susceptible to this "thermal activation" and the most likely explanation was considered to be base slippage to remove existing unpaired regions. The analogy with the $(z^8I, I)_n$ copolymer appears quite strong.

4.3.3 The effect of salt washing on interferon induction in MG63 cells

As was noted above, when cell cultures were incubated at 4° with $I_n.C_n$ and subsequently treated with 1 M sodium chloride, the interferon response was not abolished, in contrast to the results of Johnston et al. (1976). The experiments reported here (Table 4.3) involved incubations with $I_n.C_n$ in serum-free medium, whilst it is clear from the work of Johnston et al. that incubations were performed

throughout in PBS. To test whether this difference in medium could be responsible for the contrasting data, inductions were carried out in serum-free medium, PBS, and PBS supplemented with Ca^{2+} (2 mM) and Mg^{2+} (0.8 mM), both with and without salt treatment. The results are shown in Table 4.6 where it was found that while the interferon response is lower in PBS treated cells, there was still some induction even after salt treatment. When PBS was supplemented with divalent cations at the concentration obtaining in the serum-free medium, the salt washing was completely unable to reduce the interferon response. When examined microscopically, the MG63 cells appeared very rounded after the extended incubation in PBS, but appeared perfectly normal after the incubation in serum-free medium or the supplemented PBS. It certainly appears that the cells may not be as physiologically competent in their interferon response when incubated in PBS, and this may be part of the explanation for the discrepancy between these results and those of Johnston et al. (1976). On the other hand, it does not seem to be the complete answer, since there was still measurable interferon production in PBS incubated cells even after salt washing. Hence, a further explanation of the effect was sought.

The observed interferon response in cells inoculated with supplemented PBS deserves some comment. The enhancing effect of Ca^{2+} ions on the interferon response induced by $I_n.C_n$ has already been recorded for MG63 cells (Meager et al., 1978) and for L cells (Booth and Borden, 1978). In the present case, Ca^{2+} supplemented PBS contains a rather higher proportion of sparingly soluble calcium phosphate than does serum-free medium, and it seems probable that enhanced aggregation of polynucleotide at the cell surface is occurring, leading to the observed inability of sodium chloride to displace it. Such aggregation is likely to allow a greater uptake of the polynucleotides than is usual (Graham and Van der Eb, 1973).

In Table 4.7, the results of experiments to determine the extent of binding of radiolabelled $I_n.C_n$ to MG63 cells in the presence and

Table 4.6

Effect of different incubation media on interferon induction by $I_n.C_n$ in MG63 cells at 4^o

Medium	1 M salt treatment	Interferon titre ($log_{10}U/10^6$ cells)
PBS	-	2.06
PBS	+	1.09
Serum-free medium	-	2.87
Serum-free medium	+	1.77
PBS (supplemented with M^{2+})	-	3.62
PBS (supplemented with M^{2+})	+	3.62

Inductions were performed at 4^o in 50 mm dishes using $I_n.C_n$ (50 µg/ml) and incubation with sodium chloride (1 M, 2 ml) for 5 minutes as described in Section 4.2.5

Table 4.7

Effect of sodium chloride washing on MG63 cell-associated radioactivity after induction by $I_n.[^{125}I]$-C_n at 4^o

Medium	1 M salt treatment	Input counts bound %	Bound polynucleotide ($\mu g/10^6$ cell)
PBS	-	0.068 (100)	0.0244
PBS	+	0.0403 (59.4)	0.0145
Serum-free medium	-	0.112 (100)	0.0402
Serum-free medium	+	0.075 (66.9)	0.027

Incubations were performed in 30 mm dishes at 4^o as described in Table 4.4. Specific activity of $I_n.[^{125}I]$-C_n = 6.47×10^4 cpm/μg. Figures in parentheses denote percentage of counts left after salt washing compared to controls.

rather lower extent at 4° (Juliano and Mayhew, 1972). Metabolic inhibitors, such as iodoacetic acid and ouabain did not affect the uptake of the RNA. That the RNA was not superficially bound was shown by competition with a very large excess of unlabelled RNA, or by cell electrophoresis (Mayhew and Juliano, 1973) when external RNA would have caused a drastic increase in cellular mobility due to its negative charge. Whether all eukaryotic cells have the ability to absorb RNA at these low temperatures is not clear.

The mechanism of uptake of such species at 4° is worthy of some consideration. It is assumed that the uptake of solutes by cells from the medium occurs by the process of pinocytosis. Two types of pinocytic vacuole have been observed (Allison and Davies, 1974). The macropinocytotic vacuole is from 0.3-2.0 μm in diameter, and is easily visible by light microscopy. The micropinocytotic vesicle is only visible in the electron microscope and typically has a diameter of 70 nm in a variety of cell types (Casley-Smith, 1969). Uptake of large liquid droplets is usually by macropinocytosis and is generally inhibited by reagents that suppress the utilisation of metabolic energy derived from either glycolysis or oxidative phosphorylation (Silverstein et al., 1977) as well as by agents that interfere with the contractile apparatus of microtubules and microfilaments (Allison and Davies, 1974). On the other hand, only relatively small species, e.g. proteins, nucleic acids or colloidal particles, can be absorbed by the process of micropinocytosis. The microvesicles appear to pre-exist at the cell membrane in a variety of cell types (Casley-Smith, 1969) and uptake of material into such vesicles appears to be a passive process. Several electron-microsopic studies have observed the fate of colloidal material such as ferritin or thorium dioxide under a variety of conditions. In no case were metabolic inhibitors or decreased temperature found to play a role in the uptake of material into these vesicles (Jennings and Florey, 1967; Casley-Smith, 1969), although the studies were not of a quantitative nature. It seems probable that RNA would be internalised by both macro- and micro-

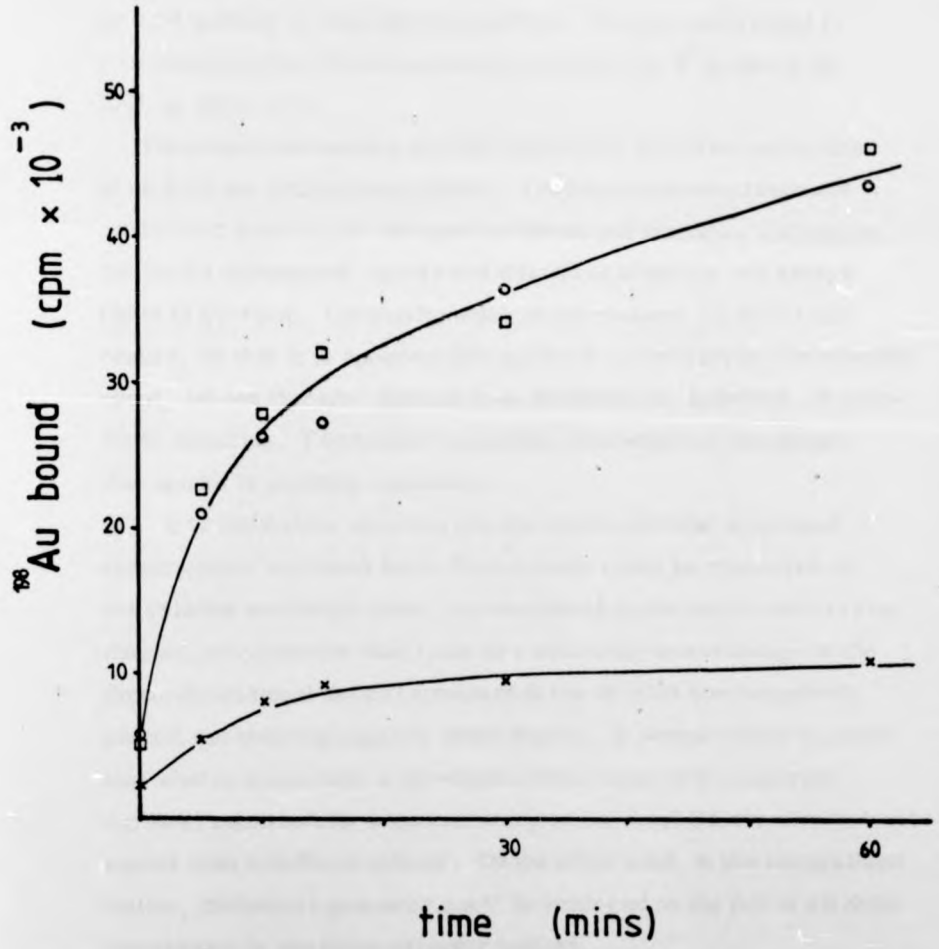
pinocytotic processes; the important point is, however, that at low temperature, the micropinocytotic process could still operate.

Quantitative studies of adsorptive micropinocytosis can be performed with radioactive colloidal gold (Davies et al., 1973) since it was found that uptake of colloidal gold was independent of cellular activity (Gosselin, 1956). The uptake of ^{198}Au by mouse peritoneal macrophages was found, however, to be inhibited at 4° C, a result somewhat at variance with prior qualitative studies on micropinocytosis. On the other hand, there was no effect of a variety of metabolic inhibitors on uptake at 37° compared with controls (Davies et al., 1973). In contrast, Stephenson et al. (1978) showed that chick embryo cells in culture readily took up the colloidal ^{198}Au at 4°, albeit at a reduced rate. Again, metabolic inhibitors had no effect, so it was assumed these cells displayed micropinocytosis. In studies with the soluble marker, horseradish peroxidase, Steinmann et al. (1974) found that uptake was markedly low at 4° (a factor of 26-fold down compared with 37°), but that over a range of temperature, the kinetics of uptake showed none of the discontinuity expected if membrane lipid phase changes were important in the uptake process.

It was decided to look at the uptake of ^{198}Au by MG63 cells at low temperature in order to assess whether micropinocytotic processes could account for irreversible uptake of $I_n.C_n$ at 4°. The results in Figure 4.9 show that in PBS and divalent ion supplemented PBS, there is a considerable accumulation of colloidal gold with time, at identical rates. The uptake in serum-free medium is rather lower, and begins to plateau, indicative perhaps that some aggregation phenomenon might be occurring in this medium. The clear result, however, is that low temperature micropinocytosis occurs in these cells. The 30 nm diameter gold particles are very similar in size to that which would be expected of 100 base-pair long RNA species, and so it seems certain that uptake of $I_n.C_n$ at 4° can occur in these MG63 cells. This conclusion would be further strengthened if advantage

Figure 4.9

Uptake of ^{198}Au by MG63 cells at 4°. Incubations in 50 mm petri dishes were performed in PBS (O – O – O), PBS supplemented with Ca^{2+} (2 mM) and Mg^{2+} (0.8 mM) (□—□—□) and serum-free medium (X – X – X). For details, see Section 4.2.7.


^{198}Au bound (cpm x 10^{-3})
50
40
30
20
10
30
60
time (mins)

could be taken of the important results of Stephenson et al. (1978) who found that chick embryo cells can be penetrated by influenza virus very efficiently even at 4^{o}. Again, it seems likely that a micropinocytotic mechanism is operative here. If MG63 cells were susceptible to infection by influenza virus, it would be a relatively simple matter to identify internalised virion markers at 4^{o}, e.g. neuraminidase, after destruction of all superficially bound virus by acid washing or immunoprecipitation. Such an experiment is considered worthwhile as supporting evidence for 4^{o} uptake of ds RNA by MG63 cells.

The causal relationship between interferon induction and uptake of ds RNA has still not been proved. The results reported here are considered some of the strongest evidence yet that when working at the limits of detection, uptake and interferon induction are always found to co-exist. Obviously uptake of non-inducer ds RNA's also occurs, so that it is apparent that uptake is a necessarily non-specific event, but one that also appears to be essential for induction of interferon induction. Two further comments lend weight to the theory that uptake is probably necessary:

(a) It is difficult to visualise how the extremely fine structural requirements exhibited by ds RNA inducer could be monitored at the cellular membrane level. As discussed in the introduction to this chapter, an alteration that leads to a relatively minor change in the three-dimensional helical structure of the ds RNA can completely abolish the inducing capacity of the duplex. It seems rather unlikely that what is essentially a two-dimensional array (the membrane surface) could be fully complementary to the overall three-dimensional aspect of an interferon inducer. On the other hand, in the intracellular milieu, the helical geometry could be exploited to the full in all three dimensions by sensitive receptor species.

(b) In what has amounted to probably the most important observation in this generally uncertain area, Marcus and Sekellick (1977) showed

that a particular type of defective interfering vesicular stomatitis virus, which contained a covalently linked +/- RNA species capable of forming a double-stranded structure, was a potent inducer of interferon when present at a ratio of one defective interfering particle per cell. This result implied that for a quantal yield of interferon, only one molecule of intracellular ds RNA per cell was required. It seems very unlikely that one molecule of ds RNA could induce interferon from a single membrane receptor. Conversely, a direct interaction with elements controlling the interferon gene could be seen as a natural explanation for this result. Although the production of interferon in this experiment was mediated by a viral inducer, and might not be considered completely relevant to the case of a synthetic inducer, it is obviously the most direct observation yet produced for the location of the triggering site, and as such provides a compelling argument for the necessity of internalisation of interferon inducers.

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APPENDIX

In the type of nearest neighbour analysis performed in this research, one of the nucleotide bases (AMP) is labelled with a 5'-[^{32}P]-phosphate. This is transferred to its 5'-neighbour upon alkaline hydrolysis of the nucleic acid, thereby permitting a quantitation of the amount of atypical base bound in this position.

The result for the sample of $(z^8A, A)_n$ described in Section 2.3.5 (base ratio z^8Ado:Ado = 1:5.03) was determined (Table 2.5) as:-

$$\frac{\text{Counts incorporated into } z^8\text{Ado}}{\text{Counts remaining in Ado}} = \frac{1}{5.75}$$

If <u>every</u> z^8Ado in the polymer was bounded on both its 5'- and 3'-side by Ado, then each z^8Ado would receive ^{32}P on alkaline hydrolysis (<u>i.e.</u> 1 part of the 5.03 originally incorporated). This would leave only 4.03 parts of the ^{32}P on the Ado residues, and the ratio of counts would be expected to be 1:4.03. If every z^8Ado occurred in dimeric structures bounded by at least one Ado on each side, <u>i.e.</u> .....p*Apz^{8}Apz^{8}Ap*A ..... (where p* represents [^{32}P]-labelled phosphate), then only one out of every two z^8Ado residues will receive ^{32}P, and the ratio of counts would be 0.5:4.53 (or 1:9.06). These are obviously artificial non-random situations, but the observed ratio of 1:5.75 allows one to state with some confidence that most z^8Ado residues occur singly, with higher order structures present to some extent.

(i) <u>Random incorporation of two nucleotides into a copolymer</u>

It is necessary to assume that the base ratio actually found in the polymer represents the probability with which incorporation occurs. Hence

$$P_Z = \frac{1}{6.03} = 0.1658$$

$$P_A = \frac{5.03}{6.03} = 0.8342$$

where P_Z and P_A represent the probability of single incorporation of z^8Ado and Ado respectively.

$P_{Z,Z} = P_Z \cdot P_Z$, $P_{Z,Z,Z} = P_Z \cdot P_Z \cdot P_Z$, etc.....

where $P_{Z,Z}$ represents the probability of two consecutive incorporations of z^8Ado, etc.

The percentage of z^8Ado residues occurring in dimers is:-

$$\frac{P_{Z,Z}}{P_Z} \times 100 = \frac{P_Z \cdot P_Z}{P_Z} \times 100 = P_Z \times 100 = 16.58\%$$

Similarly, the percentage of z^8Ado occurring in oligomers of length n is

$$\frac{P_{Z,Z,\ldots}}{P_Z} \times 100 = \frac{P_Z^n}{P_Z} \times 100 = P_Z^{n-1} \times 100$$

Now the value $P_{Z,Z}$ includes z^8Ado residues existing not only in dimers but in all higher order structures that necessarily contain such dimers. Hence the <u>actual</u> percentage of z^8Ado residues that exists in dimers alone is calculated by subtracting the percentage existing in trimers and greater, <u>i.e.</u>

$$P_Z \times 100 - P_Z^2 \times 100 = 100\, P_Z(1-P_Z)$$

or in general, the percentage of z^8Ado residues existing in oligomers of length n is:-

$$100\, P_Z^{n-1}(1-P_Z)$$

It is clear from the foregoing that 83.42% of z^8Ado residues occur singly, and will each receive a [^{32}P]-phosphate on alkaline hydrolysis. To these must be added the percentage that occur in the 3'-positons of the z^8Ado oligomers.

$\therefore$ total percentage of z^8Ado to receive [^{32}P]-phosphate

$$= 100\, P_Z^0(1-P_Z) + \frac{100P_Z^1(1-P_Z)}{2} + \frac{100P_Z^2(1-P_Z)}{3} \ldots\ldots\ldots$$

$$= 100(1-P_Z)\left(P_Z^0 + \frac{P_Z^1}{2} + \frac{P_Z^2}{3} + \frac{P_Z^3}{4} \ldots\ldots\ldots\right)$$

where the coefficients 1/2, 1/3, 1/4 etc. are required to correct for only one residue at the 3'-end of an oligomeric sequence of z^8Ado.

When calculated for z^8Ado sequences up to decamers, the percentage of z^8Ado that will pick up [^{32}P]-phosphate = 91.21%

$\therefore$ 5.03 - 0.9121 = 4.1179 parts of ^{32}P remain associated with Ado

The theoretical ratio of counts between Ado and z^8Ado would then be

4.1179:0.9121 or 4.51:1

compared to 5.75:1 found by experiment.

For a 435 base long polymer, a base ratio of 5.03:1 means that 72.14 residues are z^8Ado. The experimentally determined nearest neighbour result shows that only 1/6.75 x 5.03 = 0.745 parts of the ^{32}P were transferred to z^8Ado, i.e. only 74.5% (or 53.76) of the z^8Ado residues were positioned on the 5'-side of Ado residues. The random statistical result calculated above shows that 91.2% (or 65.8) of z^8Ado residues should be so positioned.

(ii) Non-random incorporation (nearest neighbour effect) of two nucleotides with a copolymer

In this situation, the probability of incorporation of a second residue is not dictated by considerations of total randomness, but is affected by the base incorporated immediately beforehand, i.e. by the nature of the last residue in the chain. To calculate the probabilities of occurrence of clusters of one type of residue, it is necessary to know the proportion of junctions between non-identical nucleotides, i.e. in the present case the mole fraction of .....z^8ApA..... junctions.

Using the nomenclature defined above, the probability of finding such sequences as .....z^8ApA.....is $P_{Z,A}$. However, in this case the incorporation of the Ado residue has a certain probability affected by the prior incorporation of z^8Ado; this is denoted as $P_{Z/A}$.

$\therefore$ $P_{Z,A} = P_Z \cdot P_{Z/A}$ (not $P_Z \cdot P_A$ as expected in (i))

The probabilities are equal to the mole fractions existing in the polymer; from the nearest neighbour results, it can be shown that the mole fraction, $M_{Z,A}$ is :-

$$M_{Z,A} = \frac{1}{6.75} \times \frac{5.03}{6.03} = 0.1236$$

As before:- $P_Z = 0.1658 = M_Z$

and $P_A = 0.8342 = M_A$ (M_Z and M_A are the mole fractions of z^8Ado and Ado in the polymer)

For consecutive incorporation of z^8Ado

$$P_{Z,Z} = P_Z \cdot P_{Z/Z}$$

$$P_{Z,Z,Z} = P_Z \cdot P_{Z/Z} \cdot P_{Z/Z} \quad \text{etc.}$$

The percentage of z^8Ado residues occurring in dimers or higher is:-

$$\frac{100.P_{Z,Z}}{P_Z} = 100.P_{Z/Z} \quad \text{or in general} \quad 100.P_{Z/Z}^{n-1}$$

The analysis is then precisely the same as for the random case, such that percentage of z^8Ado residues existing in oligomers of length n is:-

$$100\,P_{Z/Z}^{n-1}\,(1-P_{Z/Z})$$

and the total percentage of z^8Ado that would receive [^{32}P]-phosphate

$$= 100(1-P_{Z/Z})\,(P_{Z/Z}^{0} + \frac{P_{Z/Z}^{1}}{2} + \frac{P_{Z/Z}^{2}}{3} + \frac{P_{Z/Z}^{3}}{4} \ldots\ldots)$$

Now $P_{Z,Z} = P_Z \cdot P_{Z/Z}$ as discussed above:

$$\therefore P_{Z/Z} = \frac{P_{Z,Z}}{P_Z} = \frac{M_{Z,Z}}{M_Z}$$

Also $M_{Z,Z} + M_{Z,A} = M_Z$

$$\therefore M_{Z,Z} = M_Z - M_{Z,A} = 0.1658 - 0.1236 = 0.0422$$

$$\therefore P_{Z/Z} = \frac{0.0422}{0.1658} = 0.2545$$

When calculated for z^8Ado up to decamers, the percentage of z^8Ado that will pick up [^{32}P]-phosphate = 86.03%.

Therefore the theoretical ratio of counts in nearest neighbour analysis would be 4.85:1 (calculated as in (i)), still quite different from that observed by experiment.

Of the 72.14 z^8Ado residues in the copolymer, the number of z^8Ado at the 5'-side of Ado would be 0.8603 x 72.14 = 62.06 compared with the 53.76 expected from the nearest neighbour analysis. Hence there is still a large discrepancy in this type of non-random treatment.

To calculate for more long range effects, i.e. next-nearest neighbour, requires data on the actual quantities of dinucleotides and trinucleotides of various types actually present in the polymer, and is consequently outside the scope of the results obtained in the present investigation.